# United States Patent [19]

## Davis et al.

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[54] **NON-IMMUNOGENIC POLYPEPTIDES**

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### Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 596,931, Jul. 17, 1975, abandoned, which is a continuation-in-part of Ser. No. 381,191, Jul. 20, 1973, abandoned.

[51] Int. Cl.$^2$ .......................... **C07G 7/00; C07G 7/02; A61K 37/26; A61K 37/48**

[52] U.S. Cl. ............................ **435/181; 260/112.5 R;** 260/112.7; 424/78; 424/94; 424/177; 424/178; 435/180

[58] Field of Search ................... 195/63, 68, DIG. 11; 424/94, 177, 178, 78; 260/112.5, 112 R, 6, 8, 112.7

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[57] **ABSTRACT**

Polypeptides such as enzymes and insulin are coupled to polyethylene glycol or polypropylene glycol having a molecular weight of 500 to 20,000 daltons to provide a physiologically active non-immunogenic water soluble polypeptide composition. The polyethylene glycol or polypropylene glycol protect the polypeptide from loss of activity and the composition can be injected into the mammalian circulatory system with substantially no immunogenic response.

**27 Claims, No Drawings**

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## NON-IMMUNOGENIC POLYPEPTIDES

### RELATED APPLICATIONS

This application is a continuation-in-part of our copending application, Ser. No. 596,931 filed July 17, 1975, which, in turn, is a continuation-in-part of our then co-pending application, Ser. No. 381,191, filed July 20, 1973, both now abandoned.

### BACKGROUND OF THE INVENTION

1. Field of the Invention
Non-immunogenic polypeptides.
2. Description of the Prior Art
The use of polypeptides in circulatory systems for the purpose of engendering a particular physiological response is well known in the medicinal arts. Among the best known polypeptides utilized for this purpose is insulin which is used in the treatment of diabetes. Another group of polypeptides to which great therapeutic potential has been attributed are enzymes of the various classes. The principal factor which has severely limited the use in therapeutics of polypeptides in particular, enzymes, has been the fact that most of these compounds elicit an immunogenic response in the circulatory system. This response being the production of antibodies to the polypeptides by the circulatory system into which they are injected. This effect has one or both of two secondary consequences. The first being the destruction of polypeptides by the antibodies so called forth, or, the second more seriously, the appearance of an allergic response.

The destruction of the polypeptide by the antibodies is believed to be responsible for the rather low residence time of insulin in the human circulatory system, hence, persons afflicted with diabetes are forced to inject themselves fairly frequently with fresh doses of insulin. In the case of enzymes not only is there a problem of destruction of the polypeptide and the subsequent negation of its physiological activity but also the most undesired elicitation of an allergic reaction.

If it were found possible to so modify polypeptides that their desired physiological activity was maintained totally or at least in substantial proportion and at the same time no immunogenic response was generated within the circulatory system, then it would be possible to utilize these most valuable compounds in the mammalian circulatory system without the aforementioned disadvantages and in the far smaller amounts than has heretofore been possible.

The problems set forth hereinabove are well recognized and various approaches have been taken in attempts to solve them. The attachment of enzymes to insoluble supports has been the subject of a great deal of work. Reviews dealing with this subject will be found in Silman and Katchalski, Ann. Rev. Biochem., 35, 873 (1966), and Goldstein, Fermentation Advances, Academic Press, New York (1969) page 391. This approach however while of academic interest does not provide injectable long-life polypeptides. Another approach which has been taken to provide polypeptides of lengthened in vivo life has been the micro incapsulation of enzymes which has been discussed in numerous articles by Chang and co-workers, namely, Science, 146, 524 (1964); Trans. Am. Soc., 12, 13 (1966); Nature, 218, 243 (1968); Can. J. Physiol. Pharmacol., 47, 1043 (1969); Canad. J. Physiol. Pharmacol., 45, 705 (1967). A further approach lay in the heat stabilization of enzymes by

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attaching carboxy methylcellulose to an enzyme such as Trypsin (Mitz and Summaria, Nature, 189, 576 (1961) and the attachment of proteases to hydrophilic carriers (Brummer, et al, Eur. J. Biochem., 25, 129 (1972). These approaches however do not provide the polypeptides in a soluble form which is the most desirable for injection and dosage control of injectable materials. A further approach has been the attachment of synthetic polymers to polypeptidal proteins. A review of this work will be found in Sela, "Advances in Immunology", 5, 30, (1966) Academic Press, New York. In this work, it has been shown that while homopolymers of amino acids are nearly all non-immunogenic, when these polymers are attached to immunogenic proteins the immunogenic activity is not masked and antibodies are produced in test circulatory systems. For example, while polyglycine itself is non-immunogenic, when attached to a protein that conjugated protein becomes a hapten. Similarly while dextran itself is slightly immunogenic when coupled to insulin the insulin-dextran coupled material is believed to become substantially immunogenic.

### SUMMARY OF THE INVENTION

There are provided by this invention peptides and polypeptides coupled to polymers which are substantially non-immunogenic and retain a substantial proportion of the desired physiological activity of the base polypeptide.

In the process of this invention a substantially straight chain polymer is modified, suitably at one end thereof, either by the alteration of the terminal group or by the addition thereto of a coupling group having activity vis a vis a polypeptide and reacting said activated polymer with the polypeptide. The protected polypeptides of the present invention are injectable in aqueous solution into the mammalian circulatory system or intramuscular and call forth substantially no immunogenic response.

### DESCRIPTION OF THE PREFERRED EMBODIMENTS

The polymers utilized for protection purposes in the procedures of the present invention possess a substantially linear ethereal or carbon carbon backbone. It has been found that utilizing branch chain polymers will give rise to substances which do generate an immunogenic response. Nevertheless a certain amount of substitution in the backbone is permissible. For example, the backbone may be substituted by alkyl groups or alkoxy groups provided that said alkyl or alkoxy groups contain less than 5, preferably 2 or less carbon atoms. Among the polymers of choice may be mentioned polyethylene glycol, and polypropylene glycol, of these polyethylene glycol is particularly preferred. It is also preferred to operate in a polymer chain length area of between 500 and 20,000 daltons, about 750 to 5,000 daltons being especially preferred.

Several modes of coupling the polymer with the polypeptide are available and will be discussed in further detail hereinbelow. It should be borne in mind however that the portions of any given polypeptide moiety which has a desirable physiological action may vary from peptide to peptide. Thus, in certain enzymes one or two amino acid residues may be principally responsible for this desirable physiological activity. In choosing a coupling agent to couple a polymer to the polypeptide it would be desirable to utilize coupling

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agents which do not have an affinity for these particular active moieties. While this is a desirable goal it is not always possible to comply absolutely with it. It is therefore necessary in individual cases to effect a compromise, that is to say, sacrifice a certain amount of activity maintenance for the granting of a substantial amount of non-immunogenicity. The final results obtained will depend not only on the coupling agent used but also the proportions of reagents and molecular weight of the polymer. Nevertheless, it has been found practical with most polypeptides to utilize between 10 and 100, suitably between 15 and 50 moles of polymer per mole of polypeptide. Utilizing these proportions it has been found that at least 15% of the physiological activity has been maintained. While the scope of the invention should not be considered limited thereto, it is generally preferred to provide conditions wherein at least 40% of the physiological activity is preserved.

The procedures of the present invention are generally applicable to peptides and polypeptides, that are of particular interest in applications involving enzymes and peptide hormones. Among the enzyme categories which may be used may be mentioned:

Oxidoreductases such as: Urate: oxygen oxidoreductase (1.7.3.3; "uricase"); Hydrogen-peroxide: hydrogen-peroxide oxidoreductase (1.11.1.6; "catalase"); Cholesterol, reduced - NADP: oxygen oxidoreductase (20-$\beta$-hydroxylating) (1.14.1.9; "Cholesterol 20-hydroxylase").

Transferases such as: UDP glucuronate glucuronyltransferase (acceptor unspecific) (2.4.1.17; "UDP glucuronyltransferase"); UDP glucose: $\alpha$-D-Galactose-1-phosphate uridylyltransferase 2.7.7.12).

Hydrolases such as: Mucopeptide N-acetylmuramylhydrolase (3.2.1.17; lysozyme); Trypsin (3.4.4.4); L-Asparagine aminohydrolase (3.5.1.1; "Asparaginase").

Lyases such as: Fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase (4.1.2.12: "aldolase").

Isomerases such as: D-Xylose ketol-isomerase (5.3.1.5; xylose isomerase) and

Ligases such as: L-Citrulline: L-aspartate ligase (AMP) (6.3.4.5).

Among the peptide hormones may be mentioned are insulin, ACTH, Glucagon, Somatostatin, Somatotropin, Thymosin, Parathyroid hormone, Pigmentary hormones, Somatomedin, Etythropoietin, Luteinizing hormone, Chorionic Gonadotropin, Hypothalmic releasing factors, Antidiuretic hormones, Thyroid stimulating hormone and Prolactin.

The resultant products from these two categories of coupling are exemplified hereinbelow. Polyethylene glycol (hereinafter PEG) has been selected as the preferred embodiment of the polymer used, but has been employed solely for purposes of illustration and not limitation. Similar products would be obtained with any of the other polymers utilized in the scope of the present invention. PEG—O—$\star$ is the terminal portion of a polymer chain where Ö is the residual oxygen from the terminal hydroxyl, similarly in PEG-NH,N is the residual terminal nitrogen of the amino group replacing the terminal hydrogen. Again with respect to the peptide moiety which is representationally illustrated below as

$$\text{www} \overset{\phi}{N}H, \overset{\phi}{N}$$

is the residual nitrogen of a labile primary amino moiety on the peptide, and in

$$\overset{A}{CO}, \overset{A}{C}$$

is the carbon atom of a labile COOH group. While the foregoing exemplification shows the peptide moiety being coupled through an amino group, and this is believed to be the most likely coupling point, the invention is not limited thereto as other labile moieties, i.e. thial might also provide a coupling locus.

In the first category there may be mentioned as a suitable coupling group cyanuric chloride or fluoride. In this procedure polyethylene glycol (hereinafter PEG) is taken up in a suitable reaction inert solvent such as a hydrocarbon solvent suitably anhydrous benzene containing a small amount of a weak base such as sodium carbonate, and cyanuric chloride added thereto. The reaction mixture is then quenched with water, insoluble material removed, followed by removal of the solvent, suitably under reduced pressure to yield 2-PEG-4-hydroxy-6-chloro-1,3,5-triazine.

The thus produced activated polymers are then reacted with a solution of polypeptide in a suitable buffer. After reaction is complete the unreacted activated polymer is removed, suitably by contacting with an gel permeation resin such as Sephadex G-50 and the protected polypeptide removed and purified in the usual manner. Since these products are to be considered as polypeptides care must be taken during the purification procedure that they not be denatured. Therefore, it is desirable either to permit them to remain in buffered aqueous solution or, if it is deemed essential, to isolate them in the solid state that such isolation be carried out by the recognized procedure such as lyophilization of the protected peptide.

Thus, as the partial structure, say,



Another suitable terminal group is the acyl azide terminal group. In the production thereof, the terminal hydroxyl of the polymer is reacted with chloroacetic anhydride and subsequently with diazomethane to yield the methyl ester of the carbomethoxy ether. Treatment with hydrazine gives the corresponding hydrazide which on treatment with nitrous acid yields the desired acyl azide.

The azido moiety of the thus produced acyl azide will react with, say, a labile amino moiety on the peptide to provide a protected peptide of the partial structure, say,

$$\text{PEG—O—CH}_2 . \overset{O}{C}O . \text{NH}\text{www}$$

In place of treatment with hydrazine there may be utilized N-ethoxy carbonyl-2-ethoxy 1,2-dihydroquinoline, EEDQ. The quinoline is eliminated to give the corresponding mixed carbonic anhydride which is a "disappearing" coupling group having as the effective coupling moiety, say, the O-PEG-methyl carbonyl moiety which attaches itself to free amino groups on the polypeptide, to provide a resulting product which has

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the same structure as that shown to be obtained in the preceeding paragraph hereof.

The succinate group may also be used as a coupling moiety. In this modification the glycol, for example, PEG or PPG, is taken up in a suitable reaction inert solvent in the presence of a mild base, such as sodium bicarbonate and treated with a dihalosuccinic anhydride such as dibromosuccinic anhydride. The thus produced, say, PEG-dihalo succinate is then available for reaction with a polypeptide yielding a protected peptide of partial structure

$$ PEG{-}O{-}CO.CH.\overset{\phi}{N}\text{\raisebox{1pt}{wwww}} $$
$$ PEG{-}O{-}CO.CH.\overset{\phi}{N}\text{\raisebox{1pt}{wwww}} $$
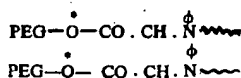
The p-diazo benzyl group is a suitable coupling agent. P-nitrobenzyl chloride is reacted with the glycol, suitably PEG in the presence of base, suitably an alkali in an anhydrous non-hydroxylic medium, preferably under reflux to produce the corresponding PEG p-nitrobenzyl ether which is then reduced to the corresponding amine by catalytic hydrogenation followed by diazotization to yield the desired O-PEG-p-diazonium benzyl ether which is then available for coupling with the polypeptide, to yield a protected peptide having the partial structure

$$ PEG{-}O{-}CH_2{-}\bigcirc{-}N_2{-}\overset{\phi}{N}\text{wwww} $$
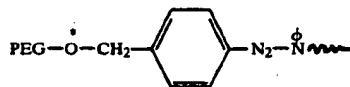
The 3-(p-diazophenyloxy)-2-hydroxy propyloxy group is prepared by treating the glycol in the presence of alkali at moderately elevated temperatures in an aqueous medium with glycidyl p-nitrophenyl ether to form the corresponding 3-(p-nitrophenyloxy) 2-hydroxypropyl ether of PEG. The nitro group is reduced to the corresponding amino group, preferably by aqueous titanous chloride in dilute mineral acid and the resulting amine diazotized to provide the diazonium ether which may then be reacted with the polypeptide, to yield a protected peptide having the partial structure

$$ PEG{-}O{-}CH_2.CH.(OH).CH_2{-}O{-}\bigcirc{-}N_2{-}NH\text{www} $$
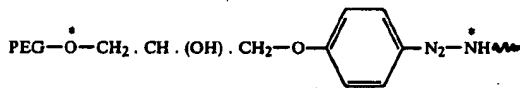
The 1-glycydoxy-4-(2'-hydroxy-3'-propyl) butane group is attached to the terminal oxygen group of the glycol and is reacted with free amino group or a polypeptide.

1,4-butanedioldiglycidyl ether is reacted with the glycol, suitably PEG in the presence of an alkali and a reducing agent such as sodium borohydride. The reaction takes place at room temperature and yields the desired PEG ether with an oxirane group at the end of the side chain. This oxirane group reacts with a free amino group to form a C—N linkage, on the polypeptide to yield a protected peptide having the partial structure

$$ PEG{-}O{-}CH_2.CH(OH)CH_2.O.(CH_2)_4.O.CH_2.CH.CH(OH)CH_2 $$
$$ \underset{\phi\,NH}{|} $$
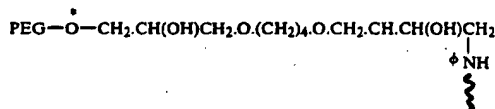
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The carboxyamino or thiocarbonylamino benzyl linkage between the terminal oxygen of the glycol such as PEG and a nitrogen of a free amino group on the polypeptide is prepared from the p-amino-benzyl ether of the glycol. The p-amino benzyl ether is treated with phosgene or thiophosgene to yield the corresponding amino acid chloride (or amino thioacid chloride) which is then reacted with the free amino group of a polypeptide, to yield a protected peptide having the partial structure

$$ PEG{-}O{-}CH_2{-}\bigcirc{-}NH.C(O\text{ or }S){-}\overset{\phi}{NH}\text{www} $$
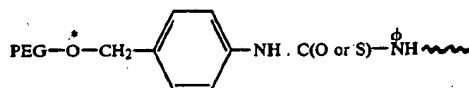
The 2-(hydroxy-3-carboxy)propyl linkage group, which is attached to the terminal oxygen of the glycol at the 2 position of the propyl group.

1,3-O-isopropylidene 2-bromo propane-1,3-diol is reacted with the glycol, say PEG in anhydrous medium in the presence of a base, suitably an alkali, preferably with heating. The resulting 2-glyceryl glycol ether is treated with cyanogen bromide at high pH and low temperature. The polypeptide is added thereto and the coupling occurs to leave the above group between the terminal oxygen of the glycol and a nitrogen of a primary amine group on the polypeptide, to yield a protected peptide having the partial structure

$$ PEG{-}O{-}CO{-}\bigcirc{-}N_2{-}\overset{\phi}{N}\text{www} $$
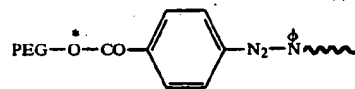
Also available is the anthranilate moiety. In this modification the glycol is again taken up in a reaction inert solvent and treated with isatoic anhydride to yield the anthranilate ester which is used without further purification in the next stage which comprises diazotization. The diazotization is carried out in the usual manner, for example, the anthranilate ester is taken up in water, the solution acidified, suitably with glacial acetic acid, cooled, and sodium nitrite added thereto. The diazonium salt thus formed is available for reaction with polypeptides.

An interesting alternative modification using azido groups involves the photochemical attachment of an azide coupling group. For example, the glycol in a buffer is treated with 4-fluoro-3-nitrophenylazide, the unreacted azide is then removed, suitably by dialysis.

The enzyme in question, for example lysozyme, is taken up in water, treated with the reagent and again, irradiated, suitably at reduced temperatures to yield, for example, PEG-2-nitrophenyl-lysozyme.

In the foregoing discussion, the carbon atom of the polymer to which the coupling moiety is attached is the carbon bearing the terminal hydroxy group. In the case of, say, PPG and PEG, two terminal hydroxy groups are present per moiety. Thus, the possibility exists of cross linking between the polypeptide moieties. Such cross linking is undesirable but may be readily avoided.

One method of cross linking avoidance is to carry out the reactions using a large excess of polymer either at the stage of combination with the coupling moiety or at the coupling state itself.

Alternatively, and preferably, one end of the polymer is pre-blocked. Such polymers, for example, alkylated PEGs such as methoxy polyethelene glycol (MPEG), are commercially available. Such polymers, of course, have only one labile group per polymer moiety.

The terminal hydroxy group may be converted into an amino group. In this procedure the polymer is re-acted at its terminal hydroxyl group either with a sulfo-nating agent such as toluene sulfonyl chloride or with a halogenating agent such as triphenyl phosphine in car-bon tetrachloride or triphenyl phosphine and a suitable N-halosuccimide. The thus produced halide or tosylate is then treated with sodium azide and reduced with lithium aluminum hydride to give the corresponding terminal amino compound.

The polymer bearing the terminal amino group is then coupled with a carboxy group of the polypeptide using methods well known in the art. The use of a water soluble carbodiimide such as 1-cyclohexyl-3-(2-mor-pholino ethyl) carbodiimide, metho-p-toluene sulfonate being especially preferred. There is thus produced an amido linkage comprising the nitrogen of the polymer and the appropriate carbonyl group of the peptide of the general structure

$$ \overset{\oplus}{PEG-NH} - \overset{\Delta}{CO} - O - \text{\sim\sim\sim} $$
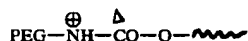
In place of direct coupling of the amino group to form an amido linkage with the polypeptide, the cou-pling may take place via a maleimide group. In this modification $\omega$-amino PEG is reacted with maleic anhy-dride and the resultant N-PEG-maleimide reacted with the desired polypeptide yield a protected peptide of the structure



Hereinabove

$$ \overset{\oplus}{-N} $$
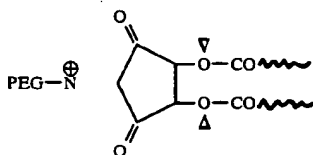
is the terminal nitrogen on the polymer moiety where the terminal hydroxyl has been replaced by a primary amino group.

Hereinafter the suffix number (i.e., PEG 750) signifies the molecular weight in daltons of the polymer in ques-tion.

In an especially preferred embodiment of this inven-tion, alkoxy, suitably methoxy polyethylene glycols, have been attached covalently to the polypeptides.

## EXPERIMENTAL

## SOURCES OF MATERIALS

Trinitrobenzene sulfonic acid was purchased from Nutritional Biochemicals Company. Cyanuric chloride (2,4,6-trichloro-s-triazine) was obtained from Aldrich Chemicals and was recrystallized twice from benzene immediately before use. Methoxy polyethylene glycols of 1900 and 5000 daltons (MPEG-1900 and MPEG-5000) were supplied by Union Carbide

## PREPARATION I

### Preparation of 2-O-Methoxy Polyethylene Glycol-4,6-dichloro-s-triazine ("Activated MPEG")

Cyanuric chloride (5.5 g; 0.03 mole) was dissolved in 400 ml anhydrous benzene containing 10 g anhydrous sodium carbonate. MPEG-1900 (19 g; 0.01 mole) was added and the mixture was stirred overnight at room temperature. The solution was filtered, and 1600 ml petroleum ether (boiling range, 35° C.–60° C.) was added slowly with stirring. The finely divided precipi-tate was collected on a filter and redissolved in 400 ml benzene. The precipitation and filtration process was repeated several times until the petroleum ether was free of residual cyanuric chloride as determined by high pressure liquid chromatography on a column of 5 $\mu$m "LiChrosorb" (E. Merck), 250×3.2 mm, developed with hexane, and detected with an ultraviolet detector. Titration of activated MPEG-1900 with silver nitrate after overnight hydrolysis in aqueous buffer at pH 10.0, room temperature, gave a value of 1.7 moles of chloride liberated per mole of MPEG.

In accordance with the above procedure but, where in place of MPEG-1900, MPEG 5000 is used, activated MPEG-5000 was similarly prepared using a 1:3 molar ratio of MPEG and cyanuric chloride.

## EXAMPLE I

### Urate: Oxygen Oxidoreductase (Uricase) (1.7.3.3)-PEG-Carbomethyl Conjugate

#### (a) Preparation of PEG

(i) Preparation of PEG-Methyl Carbomethoxy Ester

PEG 750 (2.0 g) is dissolved in liquid ammonia (30 ml.) and the solution treated with sodium until the blue color persists for 5 minutes. The ammonia is allowed to evaporate on a stream of dry nitrogen. The residue is treated with methyl chloroacetate (5 ml.) and the mix-ture allowed to stand overnight at room temperature and finally heated to 100° for 1 hour. The excess reagent is removed under reduced pressure to provide PEG-methyl carbomethoxy ester.

(ii) Preparation of PEG-Methoxy carbohydrazide

PEG-methyl carbomethoxy ester (2.0 g), methanol (300 ml.) and hydrazine hydrate (15 ml.) are refluxed overnight and the solution is evaporated under reduced pressure to yield PEG-methoxy carbohydrazide.

(iii) Preparation of PEG-Carboxymethyl Azide

The above hydrazide (1.0 g) is dissolved in 2% hy-drochloric acid (150 ml.) and 5% sodium nitrite solution (9 ml.), slowly added with stirring and allowed to stand for 20 minutes at room temperature to yield a solution of PEG-carboxymethyl azide which is used in the cou-pling stage.

#### (b) Coupling of Urate: Oxygen Oxidoreductase with PEG-Carboxymethyl Azide

The solution containing the azide (16 ml., as pro-duced in part (iii) supra) is adjusted to pH 8.7 by the addition of sodium phosphate. Uricase (25 mg) is added and the solution is stirred for 2 hours at room tempera-ture. The solution is dialyzed and the modified enzyme isolated by chromatography on Sephadex G-50. If de-sired lyophilization yields the protected enzyme in dry form.

In accordance with the foregoing procedures, but utilizing asparaginase or insulin in place of uricase there

is obtained the corresponding PEG-asparaginase or PEG-insulin conjugate. Similarly, there may be used PPG in place of PEG to provide the corresponding PPG-uricase and asparaginase conjugates.

## EXAMPLE II

Hydrogen Peroxide: Hydrogen-Peroxide Oxidoreductase (1.11.1.6; "Catalase")-2-PEG-4-Hydroxy-1,3,5-Triazin-6-yl Conjugate

### (a) Preparation of PEG-4-hydroxy-6-chloro-1,3,5-triazine

PEG 750 (30 g., 0.04 mole) or PEG 2,000 (80 g., 0.04 mole) is dissolved in 150 ml. anhydrous benzene containing 8 g. Na₂CO₃. The solution is cooled to 10° and cyanuric chloride (7.38 g., 0.04 mole) is added. The solution is stirred overnight at 10°. Water (5 ml.) is added, and the solution then is brought to room temperature for several hours, followed by heating at 40° overnight. Insoluble material is centrifuged off, and solvent is removed by reduced pressure in a rotary evaporator at 40°. A small amount of precipitate which sometimes appears during concentration is removed by the addition of a small amount of benzene to lower the viscosity, followed by centrifugation and reconcentration. The PEG-4-hydroxy-6-chloro-1,3,5-triazine, a viscous liquid at 40°, is stored in the freezer.

### (b) Preparation of PEG-HTA-Catalase Conjugate

Catalase (60 mg.; $8.7 \times 10^{-7}$ moles) is dissolved in 3 ml. 0.05 M borate buffer, pH 9.0. PEG 750 (470 mg) is added. After 3 hours the pH is readjusted to 9.0 with sodium hydroxide and the solution left at room temperature overnight. The pH is again adjusted to 9.0. Unreacted PEG is removed by passing the solution through a column of Sephadex G-50. The PEG-HTA-catalase conjugate is concentrated on a rotary evaporator to 1 mg. protein/ml. and stored in the freezer. HTA = 4-hydroxy-1,3,5-triazin-6-yl).

In accordance with the foregoing procedure, but using Carbowax 2000 a similar coupled product is obtained. Similarly, in accordance with the above procedure, but in place of catalase, D-Xylose ketol isomerase (xylose isomerase) or insulin is used, there are obtained the corresponding PEG-HTA-xylose isomerase and PEG-HTA-insulin conjugates.

## EXAMPLE III

Cholesterol, reduced - NADP: Oxygen Oxidoreductase (20-β-hydroxylating) (1.14.1.9; cholesterol 20-hydroxylase)-PEG-N-Maleimido Conjugate

### (a) Formation of N-PEG-maleimide

Maleic anhydride (1.0 g., 1/100 mole), benzene (50 ml.) and ω-amino-PEG (1/200 mole) are refluxed for 2 hours. The solution is evaporated under reduced pressure and heated at 200° in a stream of dry nitrogen for 2 hours.

### (b) Reaction of N-PEG maleimide with cholesterol 20-hydroxylase

Cholesterol 20-hydroxylase (25 mg) is added to a solution of N-PEG-maleimide (70 mg) in 0.1 M phosphate buffer (pH 7.0, 10 ml.). The solution is allowed to stand at room temperature for 1 hour. The solution is dialyzed and the desired product is isolated from the dialysate by chromatography on Sephadex G-50 to

yield a solution of the enzyme-PEG conjugate which may, if desired, be lyophilized.

In accordance with the above procedure, but where in place of cholesterole 20 hydroxylase, insulin is used there is obtained the corresponding PEG-N-maleimido insulin conjugate.

## EXAMPLE IV

UDP glucuronate glycuronyltransferase (acceptor unspecific) (2.4.1.17; "UDP glycuronyltransferase") PEG-succinato conjugate

### (a) Preparation of PEG-dibromo succinate

PEG (1.0 g) is dissolved in dry benzene (10 ml.) containing sodium bicarbonate (1.0 g). Dibromo succinic anhydride (0.5 g) is added and the solution stirred overnight. The solution is filtered and the filtrate concentrated under reduced pressure to yield PEG-dibromo succinate.

In accordance with the foregoing procedure, but using diodo succinic anhydride in place of dibromo succinic anhydride, there is obtained the corresponding PEG-diodo succinate.

### (b) PEG succinato UDP Glucuronyl transferase

UDP glucuronyl transferase (50 mg) in buffer solution (10 ml., pH 7.0) is slowly treated with PEG-dibromo succinate (100 mg) in water (5 ml) at room temperature. The pH is maintained between 7-8. The desired PEG-enzyme conjugate is isolated by chromatography, on Sephadex G-50. If desired, the product may be isolated by lyophilization.

In accordance with the foregoing procedure but where in place of UDP glucuronyltransferase there is used insulin, there is obtained PEG succinato insulin.

## EXAMPLE V

UDP Glucose: α-D-galactose-1-phosphate uridylytransferase (2.7.7.12) (UDP-GPU-transferase)-PEG-anthranilate conjugate

### (a) Preparation of PEG anthranilate ester

PEG (1.0 g) is dissolved in dry benzene (10 ml.) containing sodium bicarbonate (1.0 g). Isatoic anhydride (0.25 g) is added and the solution stirred overnight. The solution is filtered and the filtrate evaporated at 40° C. under reduced pressure to yield PEG-anthranilate ester which is used in the next step without further purification.

### (i) Preparation of PEG anthranilate ester diazonium salt

The PEG-anthranilate ester produced as above is dissolved in water (5.0 ml.) and glacial acetic acid (0.5 ml.) added, the solution is cooled to 0°-2° and a concentrated solution of sodium nitrite added dropwise with stirring. Addition of sodium nitrite is stopped when nitrous acid is present.

### (b) Coupling of UDP glucose: α-D-galactose-1-phosphate uridylyltransferase with PEG-anthranilate ester diazonium salt

UDP-GPU transferase (25 mg) is dissolved in buffer solution (5 ml., pH 7-7.5) and the solution cooled to 0°-2°. This solution is added dropwise to the diazotized solution prepared as above. The pH is maintained at 7-7.5. After 2 hours, the solution is allowed to come to room temperature and filtered and the desired compound is isolated by Sephadex chromatography. If de-

sired, the conjugate may be isolated in solid form by lyophilization.

In accordance with the above procedure but where in place of UDP-GPU transferase there is employed insulin, the corresponding insulin - PEG anthranilate ester is obtained.

### EXAMPLE VI

Mucopeptide N-acetylmuramylhydrolase (3.2.1.17)-4-azido-2-nitro-phenyl PEG

#### (a) 4-azido-2-nitrophenyl PEG

PEG (1.0 g) is dissolved in borate buffer, pH 9.8 (100 ml.) and the solution treated with 4-fluoro-3-nitrophenyl azide (1.0 g) in acetone (10 ml.). The reaction mixture is stirred at 40° overnight and filtered. The filtrate is dialyzed against water, filtered and freeze dried.

#### (b) Photochemical linkage of lysozyme with 4-azido-2-nitrophenyl PEG

Lysozyme (60 mg) is dissolved in 3 ml. of water and 4-azido-2-nitrophenyl PEG (50 mg) added. The solution was irradiated for 18 hours at 40° C. with two 125 W tungsten lamps immersed in a solution of sodium nitrite (to remove any radiation of shorter wave length than 400 nm.). The preparation was purified by chromatography on Sephadex G-50 to yield PEG-2-nitrophenyl-lysozyme conjugate upon elution. If desired, the solid conjugate may be isolated by lyophilization.

In accordance with the above procedures but where in place of lysozyme there is used insulin, there is obtained PEG-2-nitrophenyl insulin.

### EXAMPLE VII

#### Trypsin-PEG amide conjugate

ω-Amino-PEG (see Example VIII) (100 mg) is dissolved in buffer (10 ml. pH 6.0). Trypsin (50 mg) is added, followed by ethyldimethylaminopropyl carbodiimide (200 mg). The pH is maintained at 6. After 1 hour the reaction is stopped by the addition of excess acetate buffer, pH 6. The conjugate is isolated by chromatography on Sephadex G-50.

In accordance with the foregoing procedure, but where, in place of trypsin there are utilized L-citrullin-L-aspartateligase (AMP) or insulin, there is obtained L-citrullin-L-aspartateligase (AMP) (6.3.4.5)-PEG amide conjugate or insulin PEG-amide conjugate.

### EXAMPLE VIII

#### Preparation of ω-Amino PEG

#### (a) Method (i) Tosylation

PEG (mol.wt. 6000) (50 g) is dissolved in toluene (400 ml.). Toluene (60 ml.) is distilled from the mixture to remove traces of moisture. The solution is cooled and anhydrouse triethylamine (5.5 ml.) is added, followed by p-toluene sulfonyl chloride (3.4 g). The solution is kept overnight at room temperature and then filtered. The filtrate is cooled to 5° and the precipitate is collected. The polymer is dissolved in absolute ethanol (200 ml.) and sodium azide (1.0 g) is added. The solution is boiled under reflux for 36 hours to yield PEGω-azide.

#### Method (ii) halogenation

The foregoing procedure for PEG-tosylate is utilized except that thionyl bromide (2.5 ml.) is used instead of p-toluene sulfonyl chloride. The solution is refluxed for 15 minutes after the thionyl bromide to yield PEG-ω bromide which in turn is similarly converted into PEG-ω-azide.

#### (b) Conversion to ω-amino PEG

To the ethanolic solution of the ω-azido PEG, Adams catalyst is added and the solution treated with hydrogen until no more hydrogen is taken up. The solution is filtered and concentrated under reduced pressure to a small volume. Dry ether (150 ml.) is added and the polymer allowed to precipitate at 3° overnight. The polymer is collected by filtration.

### EXAMPLE IX

Fructose-1,6-diphosphate D-glyceraldehyde-3-phosphatelyase (4.1.2.12; "aldolase")-PEG amide

The enzyme (30 mg) is dissolved in 2 M sodium acetate (3 ml.), 0.1 M sodium glyoxylate (1.5 ml.) and 10 MM copper sulfate (1 ml.) Enough 0.4 M acetic acid is added to bring the pH to 5.5. The solution is kept at 20° for 1 hour. The modified protein is isolated by gel filtration on Sephadex. The modified protein is incubated with 2 M sodium acetate (3 ml.) and 2 M acetic acid (1 ml.) and ω-amino-PEG (100 mg) at 37° overnight. The desired product is isolated by chromatography on Sephadex G-50.

### EXAMPLE X

Insulin PEG-4-Hydroxy-1,3,5-Triazin-6-yl Conjugate

#### (a) Preparation of PEG-4-hydroxy-6-chloro-1,3,5-triazine

PEG 750 (30 g., 0.04 mole) or PEG 2,000 (80 g., 0.04 mole) is dissolved in 150 ml. anhydrous benzene containing 8 g. $Na_2CO_3$. The solution is cooled to 10° and cyanuric chloride (7.38 g., 0.04 mole) is added. The solution is stirred overnight at 10°. Water (5 ml.) is added, and the solution then is brought to room temperature for several hours, followed by heating at 40° overnight. Insoluble material is centrifuged off, and the solvent is removed by reduced pressure in a rotary evaporator at 40°. A small amount of precipitate which sometimes appears during concentration is removed by the addition of a small amount of benzene to lower the viscosity, followed by centrifugation and reconcentration. The PEG-3-hydroxy-6-chloro-1,3,5-triazine, a viscous liquid at 40°, is stored in the freezer.

#### (b) Preparation of PEG-HTA-Insulin Conjugate

Insulin 10 mg. i.u. is dissolved in 1 ml. 0.1 M borate buffer, pH 9.2. PEG-HTA 2,000 (179 mg) is added. After 2 hours unreacted PEG-HTA is removed by passing the solution through a column of Sephadex G-10 and adjusted. The PEG-HTA-insulin conjugate is concentrated on a rotary evaporator to protein/ml. and stored in the freezer. (HTA=4-hydroxy-1,3,5-triazin-6-yl).

In accordance with the foregoing procedure, but using PEG-750, a similar product is obtained.

In accordance with the foregoing procedure, but carrying out the reaction at pH 8.5 and at pH 10, similar products were obtained.

In accordance with the above procedure but where, in place of insulin there are used other peptide hormones including ACTH, Glucagon, Somatostatin, Somatotropin, Thymosin, Parathyroid hormone, Pigmentary hormones, Somatomedin, Erythropoietin, Lutein-

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izing hormone, Chorionic gonadotropin, Hypothalmic releasing factors, Antidiuretic hormones, Thyroid stimulating hormone and Prolactin.

### EXAMPLE XI

#### O-PEG-(P-azo insulin benzyl)ether

##### (a) Formation of O-PEG-p-amino benzyl ether

p-Nitrobenzyl chloride (3.46 g, 1/50 mole), powdered sodium hydroxide (2.0 g), anhydrous tetrahydrofuran (20 ml) and PEG (1/100 mole) are refluxed for 3 hours. The solution is filtered and evaporated under reduced pressure and PEG-p-nitronenzyl ether is precipitated by the addition of petroleum ether (bp 30°-40°). The nitro ether is reduced with hydrogen at atmospheric pressure in the presence of Raney nickel catalyst (approx. 1 g) in ethanol (50 ml). The catalyst is removed and the filtrate evaporated to give the title compound.

##### (b) Coupling with Insulin

O-PEG-p-aminophenyl ether is diazotized in aqueous solution at 0° with nitrous acid. To the purified diazotized solution insulin solution (0.25%) is added and the mixture is kept at 0° for 2 hours. The solution is dialysed at 5°-10° to yield the desired product.

In accordance with the above procedure, but using glucose oxidase (1.1.3.4) in place of insulin, there is obtained the corresponding O-PEG(p-azoglucose oxidase benzyl) ether.

### EXAMPLE XII

#### O-PEG-[p-azo insulin-(2-hydroxy propyloxy)phenyl]ether

##### (a) 3(p-Amino-Phenyloxy)-2-hydroxypropyl ether of PEG

PEG (10 g) is treated at 50° with aliquots (20 ml) of 10% aqueous glycidyl p-nitrophenyl ether and with portions (10 ml) of 10% aqueous sodium hydoxide. After 36 hours the solution is neutralized with 2 N acetic acid and evaporated. The PEG ether is extracted from the residue with benzene and precipitated by the addition of excess petroleum ether (bp 30–40).

##### (b) Coupling with Insulin

The PEG ether is reduced with a solution of titanous chloride (5%) in hydrochloric acid (6 N, 200 ml) for 5 min. at 100°. The solution evaporated at low temperature and the amino ether extracted with acetone and the ether precipitated with excess petroleum ether.

The PEG amino ether derivative (100 mg) in hydrochloric acid (5 ml, 2 N), is cooled to 0° and sodium nitrite solution (2%) added. The pH is adjuted to 7.6–7.7 (phosphate buffer) and aliquots of insulin solution (0.5%) added at 0°. After 18 hours at 0°-5° the solution is dialyzed to yield the desired product. In accordance with the foregoing procedures but using amglase in place of insulin, there is obtained the corresponding O-PEG[azo-α-amylase-(2-hydroxy propyloxy)phenyl] ether.

### EXAMPLE XIII

#### O-PEG Methyl carboxy Insulin

##### (a) Activation of PEG

To a solution of O-PEG-methyl carbomethoxy ether (Ex. 1) (1.0 g) in water (10 ml) is added dropwise a solution of 1.0 g of N-ethoxycarbonyl-2-ethoxy 1,2-

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dihydroquinoline (EEDQ) in 10% acetone (10 ml.). The pH is maintained at 7.0. After 30 minutes, the pH is adjusted to 1.0 with concentrated hydrochloric acid and is maintained at this pH for 90 seconds to destroy excess EEDQ. The solution is then adjusted to pH 8.

##### (b) Coupling to Insulin

Insulin (50 mg) in phosphate buffer (pH 8.0) was added to the solution of the activated PEG at 4°-5°. After ½ hour the solution was dialyzed against water to yield the desired product.

In accordance with the above procedure but using urease (3.5.1.5) in place of insulin, there is obtained the corresponding O-PEG methylcarboxy urease.

### EXAMPLE XIV

#### 2-O-PEG-1-Hydroxy-3-carboxy Insulin propane

##### (a) 2-Glycerol ether of PEG

PEG (10 g) and 1,3-O-isopropylidene-2-bromo-propane-1,3-diol (10 g), powdered sodium hydroxide (15 g) are heated with stirring in tetrahydrofuran (50 ml) for 3 hours. The solution is filtered and evaporated. The residue is dissolved in 0.1 N hydrochloric acid and the solution allowed to stand at room temperature overnight. The solution is neturalized and evaporated. The PEG ether is taken up in acetone and precipitated by the addition of excess petroleum ether.

##### (b) Coupling with Insulin

The 2-glycerol ether of PEG (200 mg) is treated with cyanogen bromide (4 ml. 0.5 M) at pH 11.5 at 0°-5° for 8 minutes. Insulin (10 mg) in 0.1 M $NaHCO_3$ is added to the above solution and the mixture is stirred at 4° C. for 24 hours. The solution is dialyzed to yield the desired product.

In accordance with the above procedure but where in place of insulin there is utilized catalase (1.11.1.6) the corresponding 2-O-PEG-hydroxy-3 hydroxy-3 carboxy catalase propane.

### EXAMPLE XV

#### 1-(Insulin-2-hydroxy propoxy)-4-3″-O-PEG-2″-hydroxy propoxy Butane

##### (a) Oxirane ether of PEG

PEG (5.0 g), 1,4-butanediol diglycidyl ether (1 ml) and 0.6 M sodium hydroxide (1 ml) solution containing 2 mg of sodium borohydride were stirred at room temperature for 8 hours. The solution was neutralized and evaporated. The residue is extracted with acetone and the PEG ether precipitated by the addition of excess petroleum ether.

##### (b) Coupling to Insulin

Oxirane-PEG (1.0 g) and trypsin (50 mg) in buffer solution (pH 8.5) were allowed to react at room temperature for 48 hours. The solution was dialyzed to yield the desired product.

In accordance with the above procedure but where in place of insulin there is utilized trypsin (3.4.4.4), there is obtained the corresponding ether.

## EXAMPLE XVI

### p-Insulin carboxy amino benzyl/PEG Ether

#### (a) p-Insulin cyanatobenzyl ether of PEG

p-Aminobenzyl ether of PEG (Ex.XI) (100 mg) in phosphate buffer (pH 6.8, 0.5 ml) is stirred at room temperature and phosgene solution (10%, 1 ml) carbon tetrachloride added over a period of 1 hour. The carbon tetrachloride is removed under vacuum and the pH of the solution adjusted to pH 8.5.

#### (b) Coupling to Insulin

Insulin (1.5% in borate buffer (pH 8.5) is added to the above solution and the mixture is stirred for 18 hours at 0°–5°. The solution is dialyzed against water to yield the desired product.

Note the thiophosgene may be substituted for phosgene. This will give the corresponding thiocarbonyl derivative.

In accordance with the above procedure but where in place of insulin there is used ACTH, the corresponding P-ACTH carboxy or thiocarbonyl amino benzyl PEG ether is produced.

## EXAMPLE XVII

The procedures of Examples I and III through XVI are carried out in the same manner except that in place of PEG tjere are utilized methoxy polyethylene glycols of 1,900 and 5,000 daltons (MPEG 1900 m MPEG 5000) to give the corresponding MPEG protected product.

## EXAMPLE XVIII

In accordance with the procedure of Example II there is utilized activated MPEG prepared in accordance with Preparation I to yield the corresponding MPEG-HTA-polypeptide conjugates.

### Enzymatic Activities of PEG-catalase

Catalase—assayed by the method of Beers and Sizer (J. Biol. Chem., 195, 133 (1952)). Both PEG 750-catalase and PEG 2,000-catalase were slightly more active than unmodified catalase:
Unmodified catalase: 41,500 units/mg. protein
PEG 750-catalase: 43,750 units/mg. protein
PEG 2,000-catalase: 43,500 units/mg. protein
The heat stabilities of unmodified and PEG 750-catalase were similar at 37° and 60°.

### Antigentic Testing of PEG-catalase

Immunization - New Zealand, white adult female rabbits were immunized by the intravenous route with a 1.5 mg/ml. stock solution of beef liver catalase for a period of 4 weeks by which time it received a total of 55 mg. of catalase. Rabbits were bled by cardiac puncture 7 and 14 days after completion of the immunization schedule. Approximately 1 month later, rabbits were injected with 5 mg. of the appropriate antigen and bled by cardiac puncture 1 week after receiving the booster injection.

### In vivo and In vitro Tests

Following the last bleeding—an area on the back of the rabbits was shaved with electric clippers and residual fur removed with depilatory. The next day rabbits were injected intradermally with 0.1 ml. of the appropriate antigen and borate-buffered saline (diluent) to test for the possible development of immediate—and/or delayed—type hypersensitivities. All animals were observed for skin reactions for a period of 96 hours after injection.

Rabbit antisera obtained after initial or booster immunization were assayed in vitro for precipitating (1) and complement fixing activities (2,3). Each antiserum (undiluted, 1/10, 1/50) was set up in gel diffusion plates (1.0% Noble Agar in physiological saline with 1.0% azide added as preservative) against both catalase and PEG-HTA-catalase solutions (concentrations ranging from 1000γ/ml. to 5γ/ml). Plates were incubated at room temperature for 48 hours and at 4° C. for 3–4 days. Complement fixing activity of the heat inactivated, sheep red blood cell absorbed antisera was determined using the Microtiter Complement-fixation test (Cooke Engineering Co., Alexandria, Virginia). Antisera were run 1:1 and 1:10 dilutions against catalse and PEG-HTA-catalase dilutions ranging from 1000γ/ml. down to 15γ/ml. Plates were read for presence or absence of hemolysis following incubation at 37° C. for 30 minutes.

### Intravenous Immunization with Catalase

#### (a) Analysis of Rabbit Antiserum after Immunization with Catalase

Gel Diffusion - Antiserum tested undiluted and 1/10 was positive for precipitating antibodies against catalase. Improtantly, when this antiserum, prepared against native enzyme, was tested against the enzyme modified by polyoxyethylene residues there was no evidence of any cross-reactions.

Complement Fixation—Both initial and booster sera were positive for complement-fixing activity when tested against native catalase. When the modified catalase was used as the reacting antigen, the results were negative.

### PEG-Insulin Immunoassay

PEG-HTA-Insulin was prepared in accordance with Example II and tested for antigenic activity by the method of

| Sample | (1) Insulin Activity, μ units/ml. (animal assay) | (2) Insulin Antibodies Activity, μ units/ml. (radioimmunoassay) |
|---|---|---|
| Unmodified Insulin | 137 | 127 |
| PEG-HTA Insulin (750) | 72* | 0 |
| PEG-HTA Insulin (2,000) | 90* | 0 |

*These figures are obtained as a dilution figure and are not actual assay figures.

The foregoing results show that PEG-HTA-insulin has no antigenic activity vis a vis insulin antiserum.

### PEG-HTA-Insulin Assay

Preliminary insulin and PEG-HTA-insulin containing the same amount of insulin were injected into test rabbits and blood sugar levels measured by the "Glucostat" method. (Worthington Biochemical Corporation, Freehold, N. J.).

| After 3 hours | Blood glucose level |
|---|---|
| PEG-HTA Insulin | 50% of normal |

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| After 3 hours | Blood glucose level |
|---|---|
| Insulin | 20% of normal |

These tests indicate that the PEG-HTA-insulin has insulin activity of about 50% of insulin based on weight of conjugated insulin administered.

### PEG Insulin Rat Assay

Standard laboratory rats (220/225 g.) were made diabetic by the procedure of Goldner (Bull.-N.Y.Acad.Med. 21, 44, (1945) utilizing alloxan injections. No food was given to the test animals during the test period. Insulin, & PEG 2000-HTA insulin formulations produced at pH 9.2 and 10.5 were administered to the test rats. The pH 10.5 product showed no lowering of blood glucose levels.

The results are tabulated below and summarized in the Figure.

| Time (hrs) | Blood Glucose Level mg/100 ml. | Drop Over Initial Level (mg/100 ml) | % Drop Over Control |
|---|---|---|---|
| Insulin (intramuscularly) | | | |
| 0 | 410 | — | — |
| 1 | 295 | 135 | 32.9 |
| 2 | 270 | 140 | 34.1 |
| 4 | 290 | 120 | 29.2 |
| 6 | 400 | 10 | 02.4 |
| Control (no food during test period) | | | |
| 0 | 520 | 0 | 0 |
| 2 | 510 | 10 | 1.9 |
| 4 | 490 | 30 | 5.7 |
| 6 | 470 | 50 | 9.6 |
| 10 | 395 | 125 | 24.0 |
| PEG HTA Insulin (pH 9.2) (intravenous) | | | |
| 0 | 305 | — | — |
| 2 | 72.5 | 232 | 76.2 |
| 4 | 101.5 | 203.5 | 66.7 |
| *5 | — | — | — |
| 8 | 230 | 75 | 24.6 |

*animals fed after this point.

We claim:

1. A physiologically active, substantially non-immunogenic water soluble polypeptide composition comprising, a physiologically active polypeptide coupled with a coupling agent to

at least one substantially linear polymer having a molecular weight of between about 500 to about 20,000 daltons selected from the group consisting of polyethylene glycol and polypropropylene glycol

wherein the polymer is unsubstituted or substituted by alkoxy or alkyl groups, said alkoxy or alkyl group possessing less than 5 carbon atoms.

2. The polypeptide composition of claim 1 wherein the polypeptide is selected from the group consisting of insulin, ACTH, glucagon, somatostatin, somatotropin, thymosin, parathyroid hormone, pigmentary hormones, somatomedin, etythropoietin, luteinizing hormone, chorionic gonadotropin, hypothalmic releasing factors, antidiuretic hormones, thyroid stimulating hormone and prolactin.

3. The polypeptide composition of claim 2 wherein the polypeptide is insulin.

4. The polypeptide composition of claim 1 wherein the polymer has a molecular weight of between about 750 and 2,000 daltons.

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5. The polypeptide composition of claim 4 which comprises between 10 and 100 polymer moieties per molecule of insulin.

6. The polypeptide composition of claim 1 wherein the polymer is polyethylene glycol.
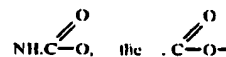
7. The polypeptide composition of claim 4 wherein the polypeptide is insulin.

8. The polypeptide composition of claim 6 wherein the polypeptide is insulin.

9. The polypeptide composition of claim 1 wherein the group between the polypeptide and the polymer has the formula selected from the group consisting of
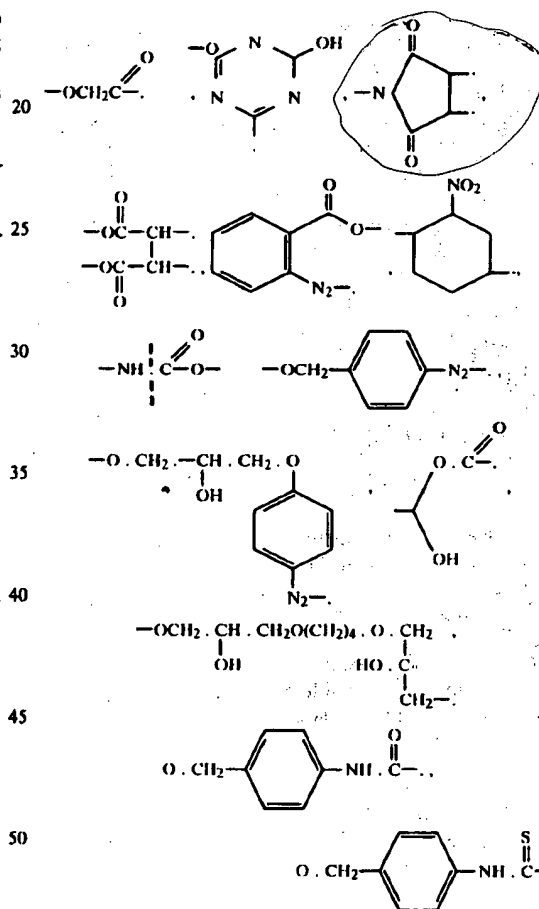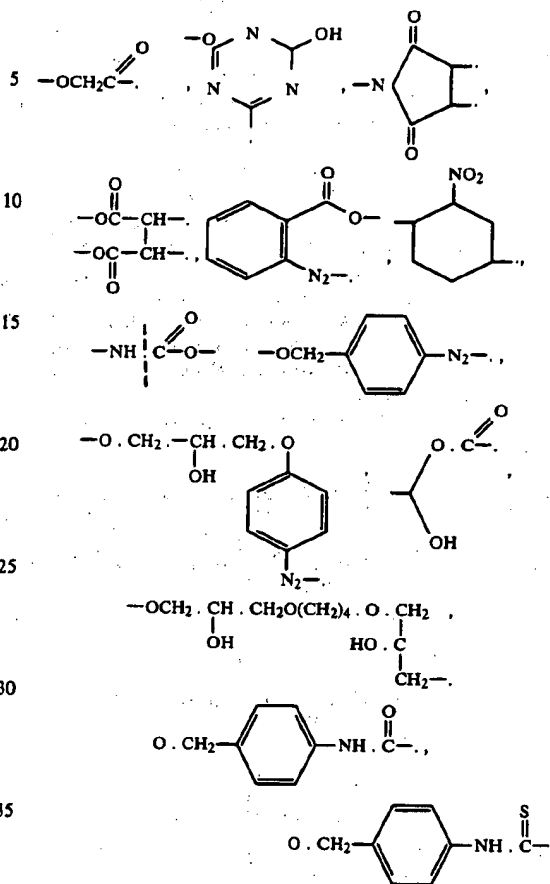


wherein the bond designated—· of each group is attached to the polypeptide provided that in the case where the group is



portion is part of the polypeptide carboxy group.

10. The polypeptide composition of claim 9 wherein the polypeptide is insulin.

11. The polypeptide composition of claim 10 wherein the group is

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**12.** The polypeptide composition of claim 11 wherein the polymer is polyethylene glycol.

**13.** A substantially non-immunogenic insulin-containing composition comprising the insulin composition of claim 3 and a pharmaceutically acceptable injectable carrier.

**14.** A process of preparing a physiologically active, substantially non-immunogenic water soluble polypeptide composition from a polypeptide; originally having physiological activity and immunogenicity which comprises the steps of:

(a) reacting at least one terminal carbon atom bearing a hydroxy group, of a substantially straight chain polymer selected from the group consisting of polyethylene glycol and polypropylene glycol having a molecular weight of from about 500 to about 20,000 daltons said polymer being unsubstituted or substituted by alkoxy or alkyl groups, said alkoxy or alkyl groups having less than 5 carbon atoms with a coupling agent to provide an activated polymer containing a reactive terminal group, and;

(b) reacting a physiologically active immunogenic poly peptide with from 10 to 100 moles of said activated polymer per mole of poly peptide by coupling said polypeptide to the reactive terminal group of the polymer to provide said water soluble polypeptide composition.

**15.** A process of claim 14 wherein the coupling agent is cyanuric chloride.

**16.** A process of claim 15 wherein the polypeptide is insulin.

**17.** A physiologically active substantially non-immunogenic water soluble enzyme composition comprising a physiologically active enzyme coupled with a coupling agent to

at least one substantially linear polymer selected from the group consisting of polyethylene glycol and polypropylene glycol having molecular weight of between 500 and 20,000 daltons

wherein the polymer moiety is unsubstituted or substituted by alkoxy or alkyl groups, said alkoxy or alkyl group possessing less than 5 carbon atoms.

**18.** The enzyme composition of claim 17 wherein the polymer has a molecular weight of between about 500 and about 5,000 daltons.

**19.** The enzyme composition of claim 17 which comprises between 10 and 100 polymer moieties per molecule of enzyme.

**20.** The enzyme composition of claim 17 wherein the polymer is polyethylene glycol.

**21.** The enzyme composition of claim 17 wherein the enzyme is selected from the group consisting of oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases.

**22.** The enzyme composition of claim 17 wherein the group between the enzyme and the polymer has the formula selected from the group consisting of



wherein the bonds designated— of each group is attached to the enzyme provided that in the case where the group is



portion is part of the polypeptide carboxy group.

**23.** A process of preparing a physiologically active, substantially non-immunogenic water soluble enzyme composition from an enzyme originally having physiological activity and immunogenicity which comprises the steps of:

(a) reacting at least one terminal carbon atom bearing a hydroxy group, of a substantially straight chain polymer selected from the group consisting of polyethylene glycol and polypropylene glycol having a molecular weight of from about 500 to about 20,000 daltons said polymer being unsubstituted or substituted by alkoxy or alkyl groups, said alkoxy or alkyl groups having less than 5 carbon atoms with a coupling agent to provide an activated polymer containing a reactive terminal group, and

(b) reacting a physiologically active immunogenic enzyme with from 10 to 100 moles of said activated polymer per mole of enzyme by coupling the enzyme to the reactive terminal group of said poly-

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mer to provide said water soluble enzyme composition.

24. The process of claim 23 wherein the enzyme is selected from the group consisting of oxidoreductases, transrerases, hydrolases, lysases, isomerases, and ligases.

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25. A process of claim 24 wherein the coupling moiety is cyanuric chloride.

26. A process of claim 24 wherein the polymer is polyethylene glycol.

27. A process of claim 26 wherein the coupling moiety is cyanuric chloride.

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# United States Patent [19]

## Kinstler et al.

[11] **Patent Number:** 5,824,784

[45] **Date of Patent:** Oct. 20, 1998

US005824784A

[54] **N-TERMINALLY CHEMICALLY MODIFIED PROTEIN COMPOSITIONS AND METHODS**

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[21] Appl. No.: **321,510**

[22] Filed: **Oct. 12, 1994**

[51] **Int. Cl.⁶** ............................... A61K 38/18; C07K 1/00
[52] **U.S. Cl.** .......................... 530/399; 530/350; 530/402; 530/410
[58] **Field of Search** .................................. 530/351, 410, 530/350, 399, 402; 424/85.1, 85.4

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[57] **ABSTRACT**

Provided herein are methods and compositions relating to the attachment of water soluble polymers to proteins. Provided are novel methods for N-terminally modifying proteins or analogs thereof, and resultant compositions, including novel N-terminally chemically modified G-CSF compositions and related methods of preparation. Also provided is chemically modified consensus interferon.

**12 Claims, 15 Drawing Sheets**



Site of Modification
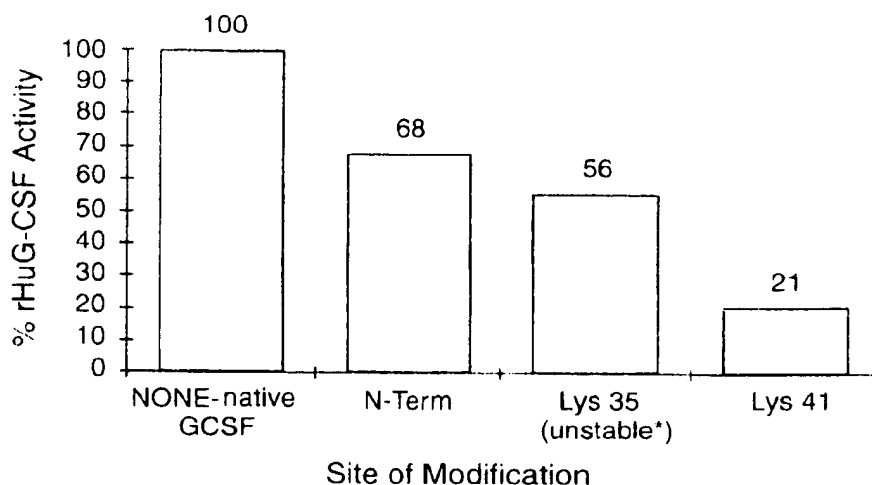
* contains de-Pegylated rHuG-CSF, generated during storage.

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Francis, Focus on Growth Factors 3, 4–10 (May 1992).

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Klein et al., J. Chromatog. 454, 205–215 (1988).

McGoff et al., Chem. Pharm. Bull. 36, 3079–3091 (1988).

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Rose et al., Bioconjugate Chemistry 2, 154–159 (1991).

Sada et al., J. Fermentation Bioengineering 71, 137–139 (1991).

Stryer, Biochemistry 2nd Ed. pp. 80, Table 4–1.

Wetzel et al., Bioconjugate Chem. 1, 114–122 (1990).

**Fig. 1A**



Fraction Number
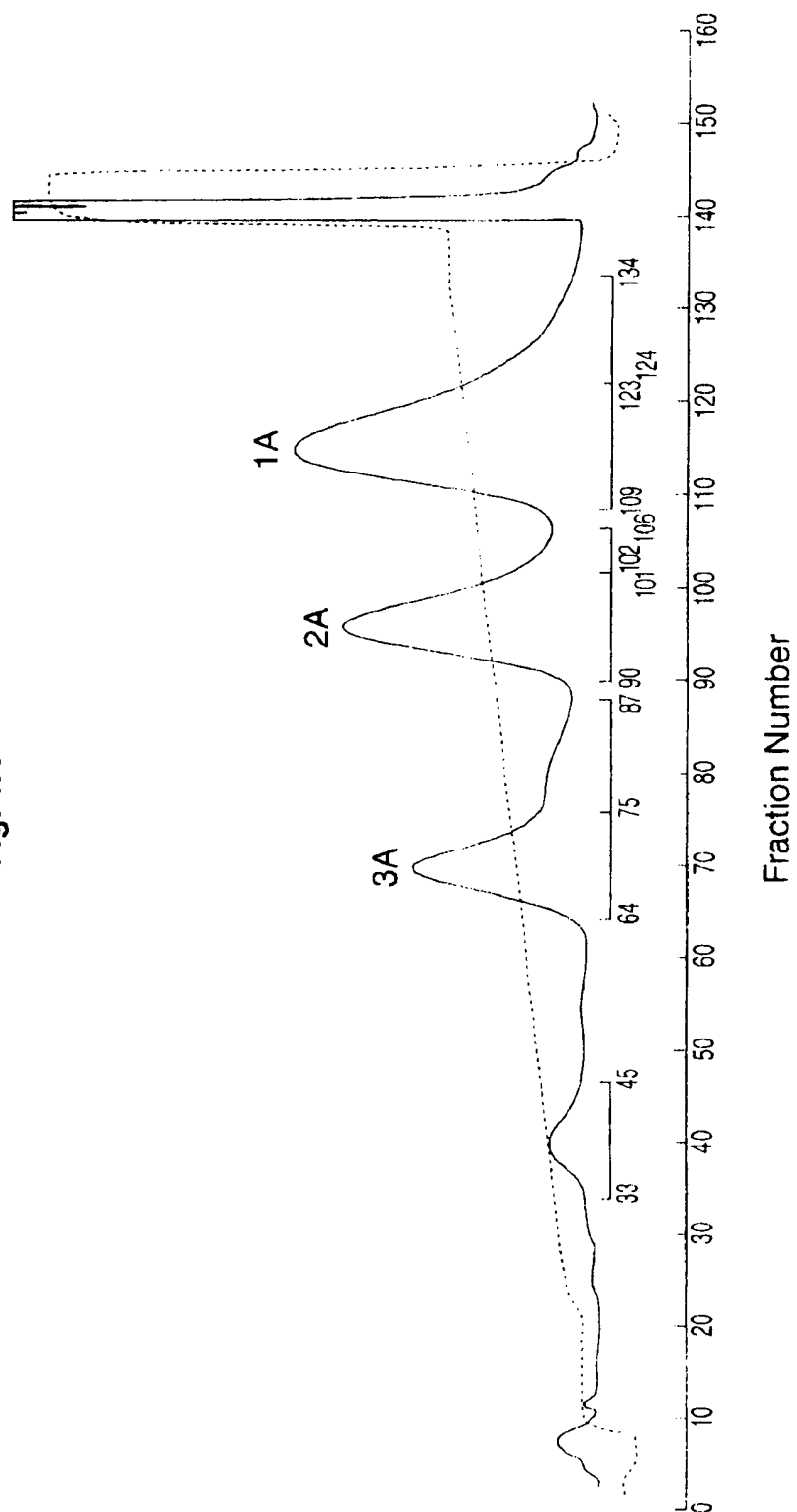
**Fig. 1B**
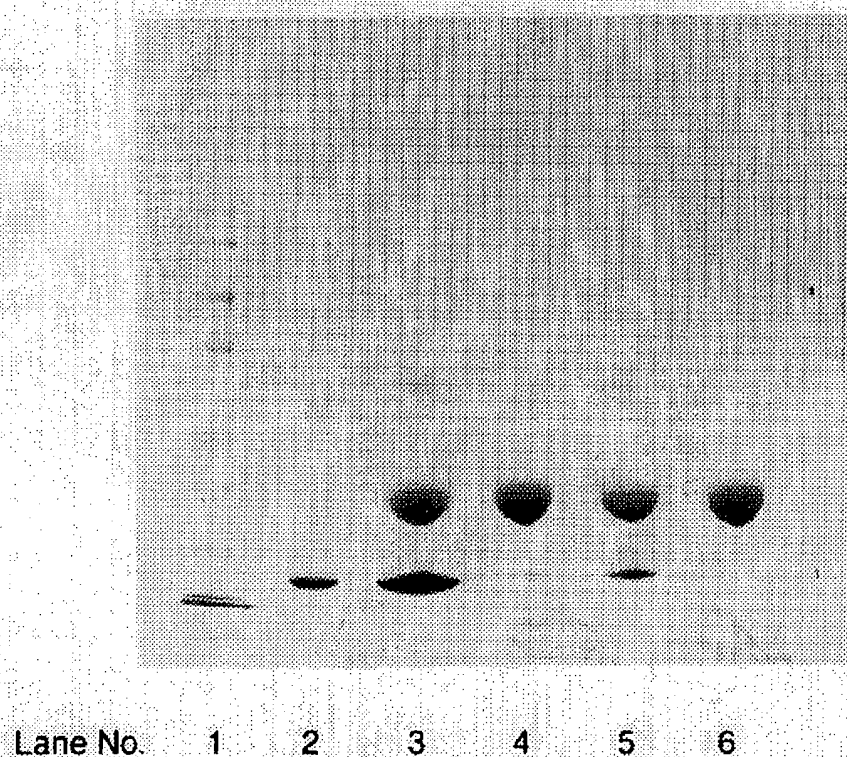


| Lane No. | Sample | ug loaded |
|---|---|---|
| 1 | MW Protein Standards | - |
| 2 | rHuG-CSF Std | 3.0 |
| 3 | SCM-PEG-GCSF Reaction Mix | 10.0 |
| 4 | Species 1 (N-Term) | 10.0 |
| 5 | Species 2 (Lys-35) | 10.0 |
| 6 | Species 3 (Lys-41) | 10.0 |

**Fig. 2**

SEC-HPLC Profiles of
(A) rHuG-CSF standard
(B) SCM-PEG-GCSF Reaction Mixture
(C) Species 1 (N-Term Derivative)
(D) Species 2 (Lys-35 Derivative)
(E) Species 3 (Lys-41 Derivative)

**Fig. 3A**

Fig. 3B

Fig. 3C



GCSF Standard
SV8 Mapping

GCSF Mono-PEG 3

66.4 min

30.3 min

Time (min.)

Scaled
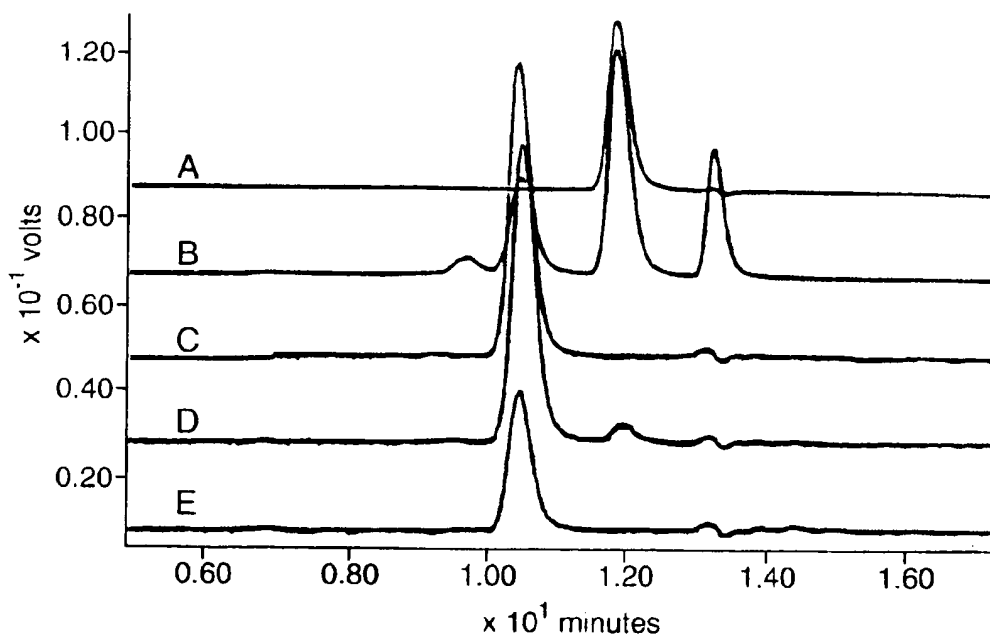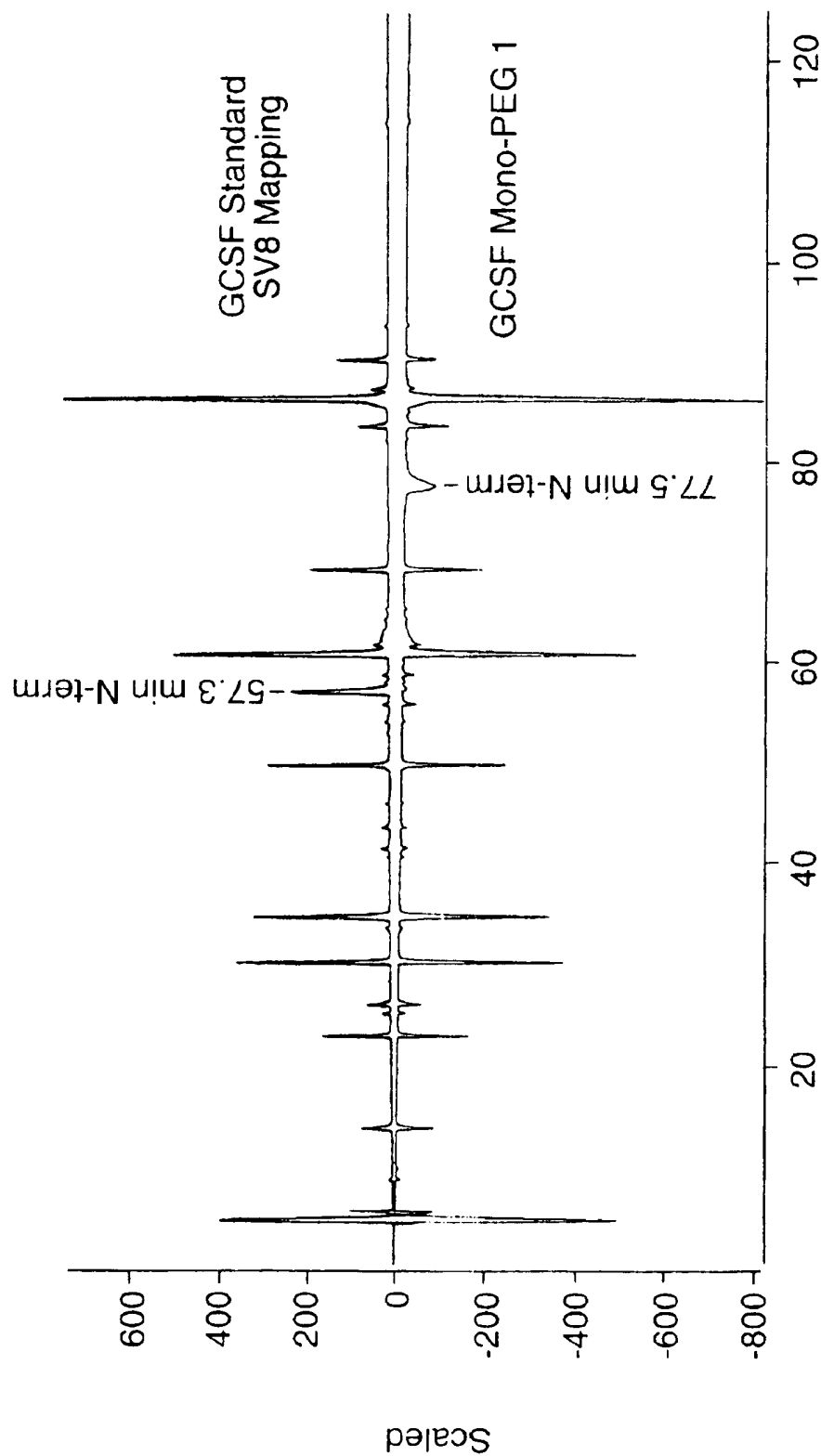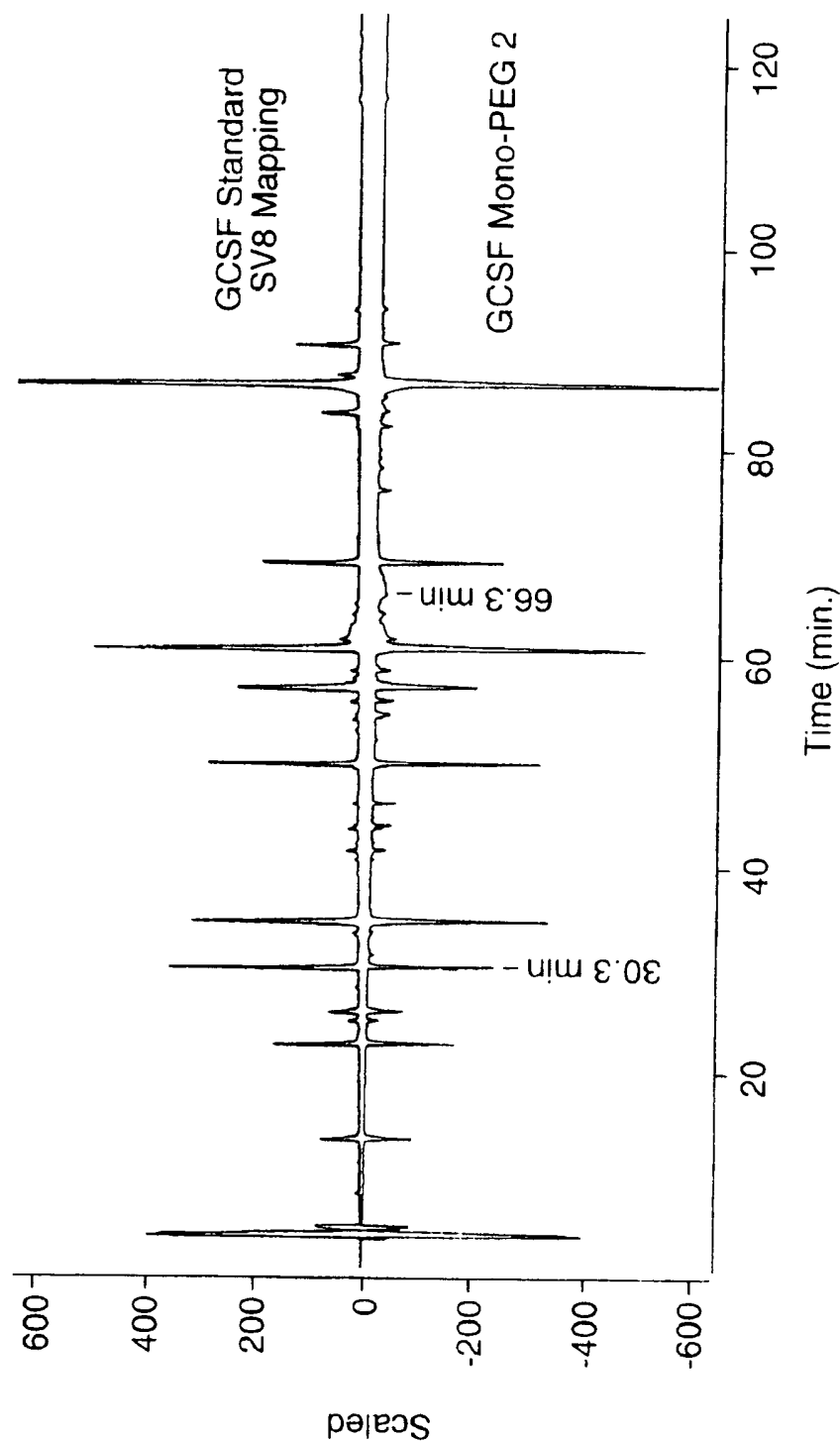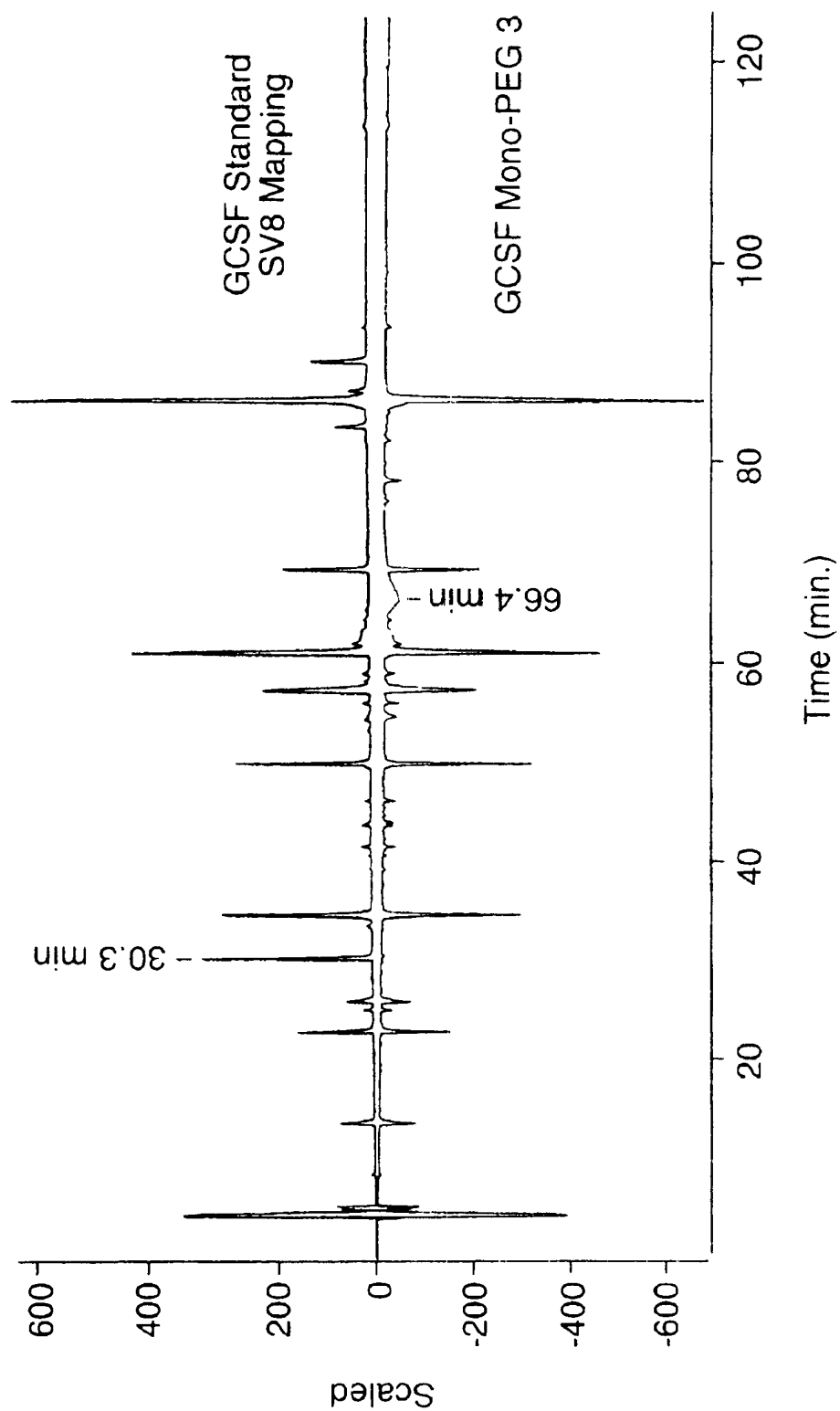
**Fig. 4**
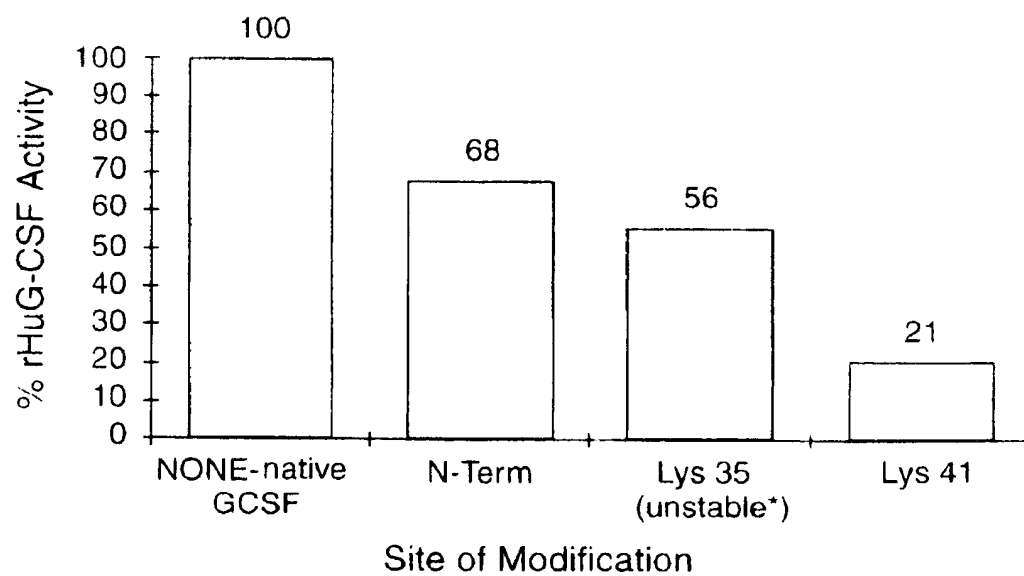


\* contains de-Pegylated rHuG-CSF. generated during storage.

Fig. 5A

**Fig. 5B**

**Fig. 6A**
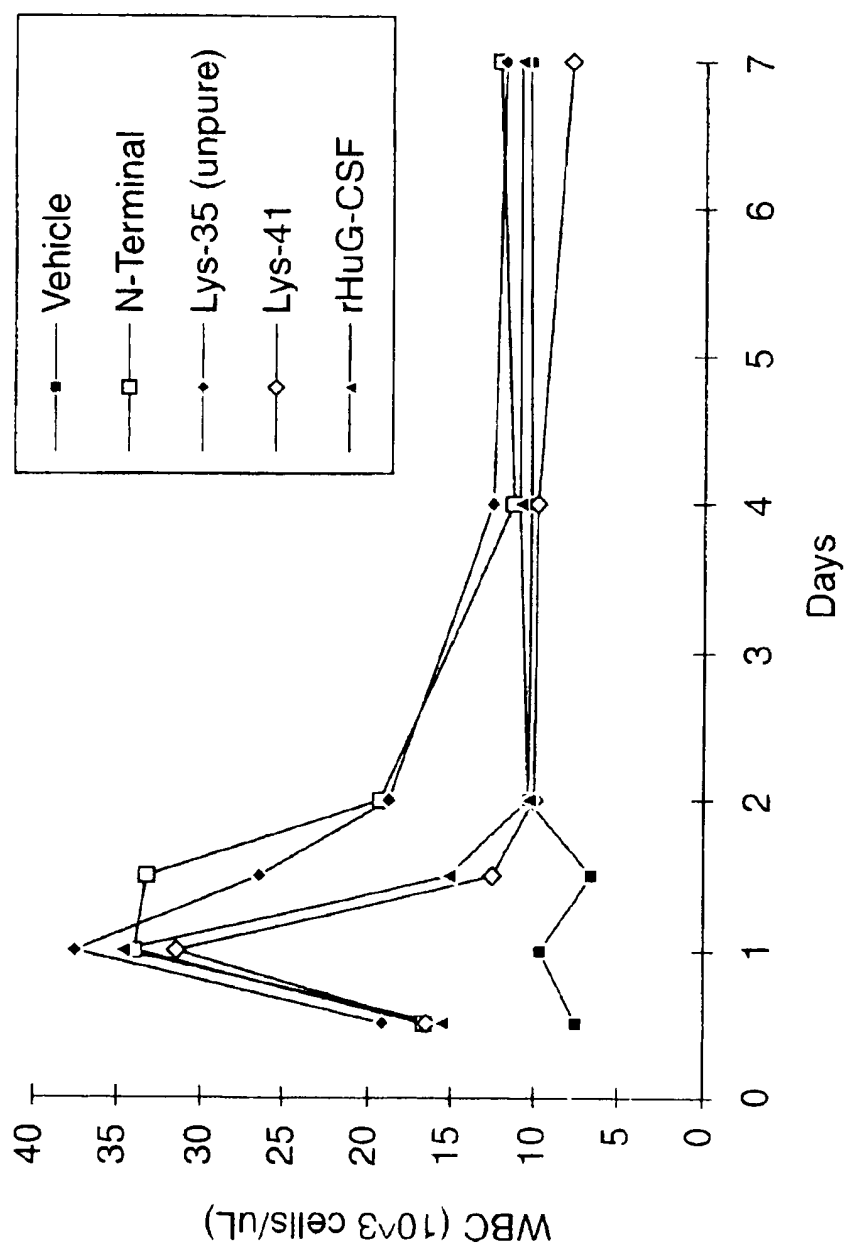
N-term monopegylated G-CSF, pH 6
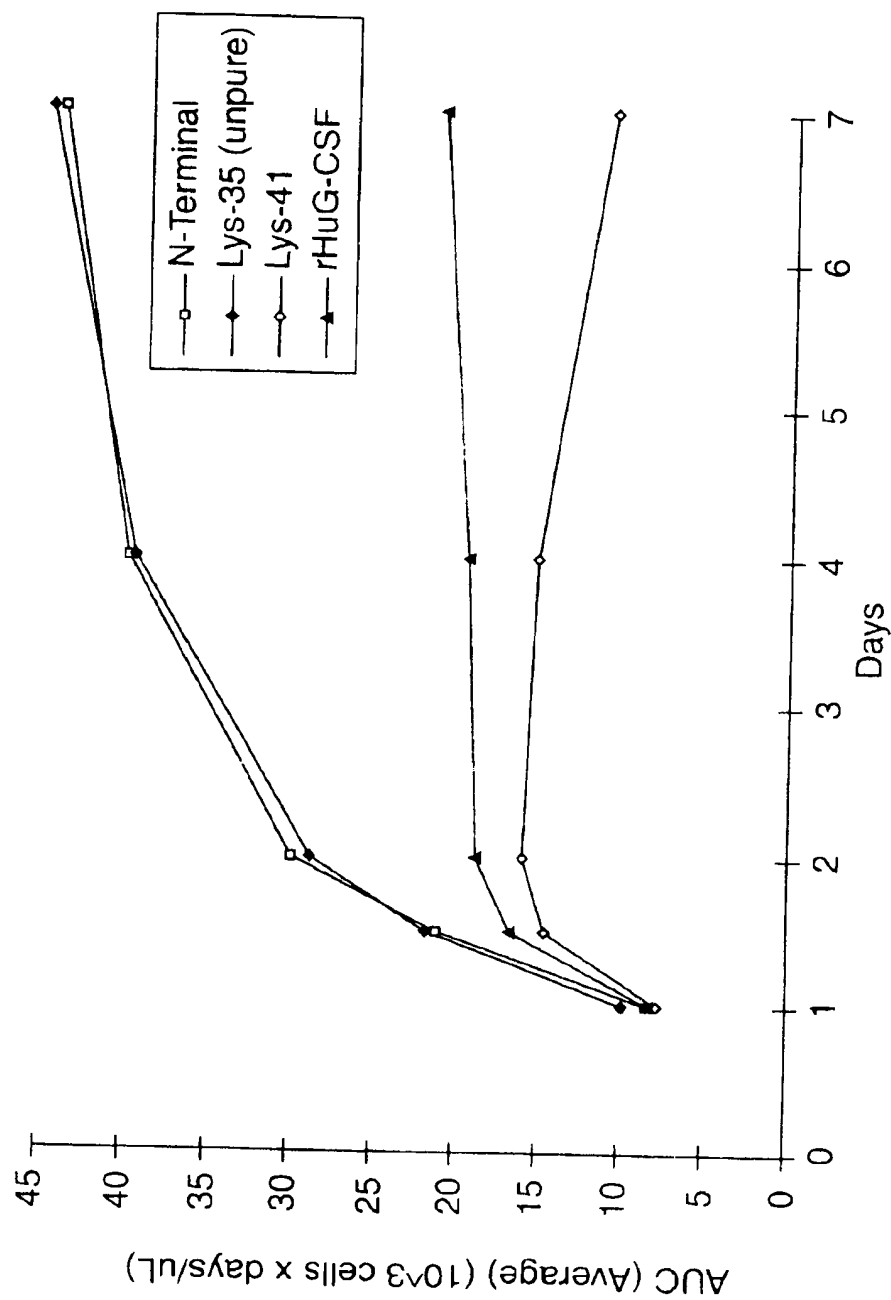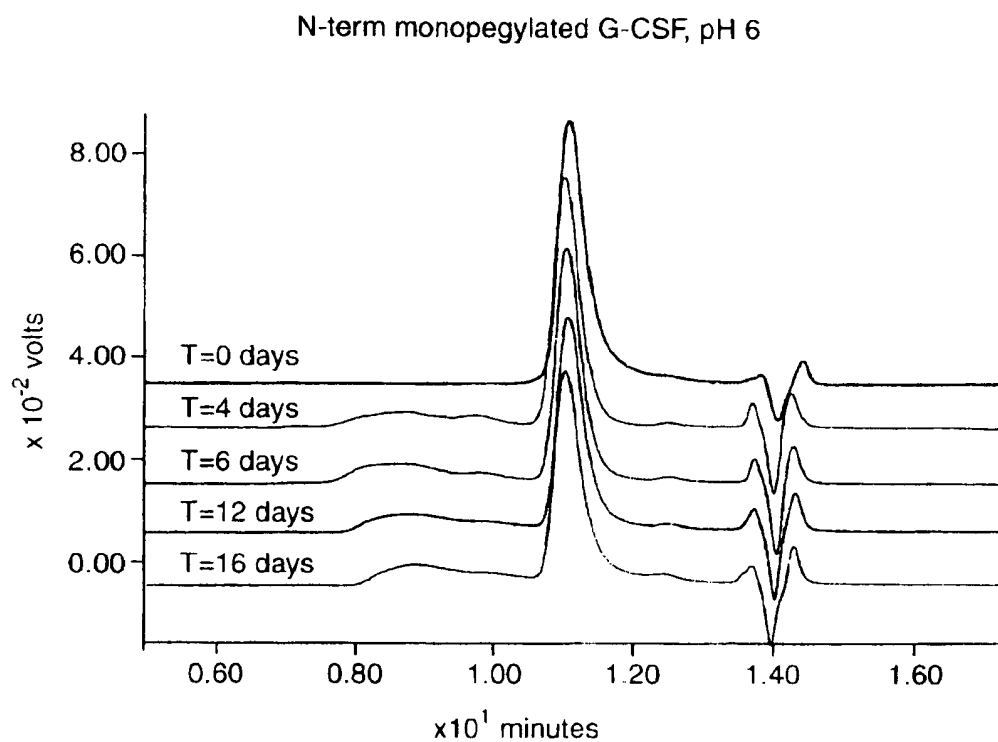
**Fig. 6B**

Lys 35 monopegylated G-CSF, pH 6
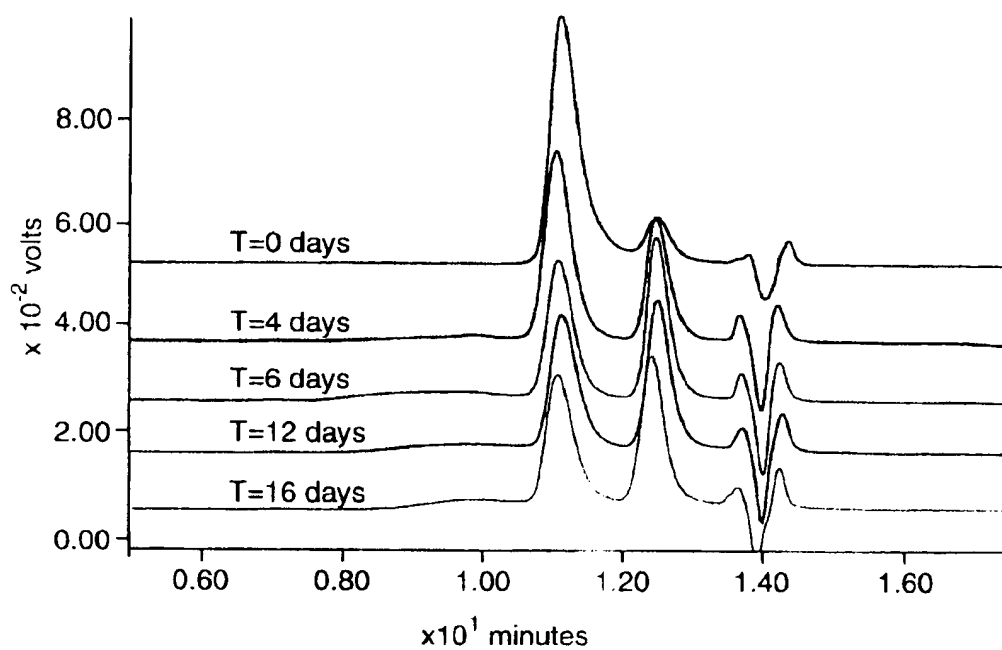
**Fig. 6C**

Lys 35 monopegylated G-CSF, pH 7
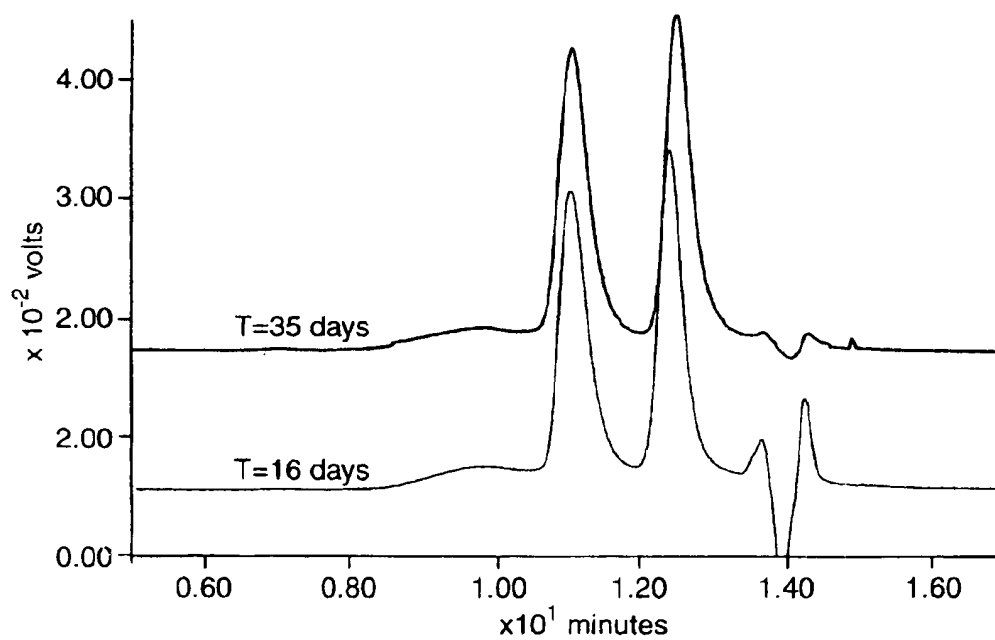
**Fig. 7**
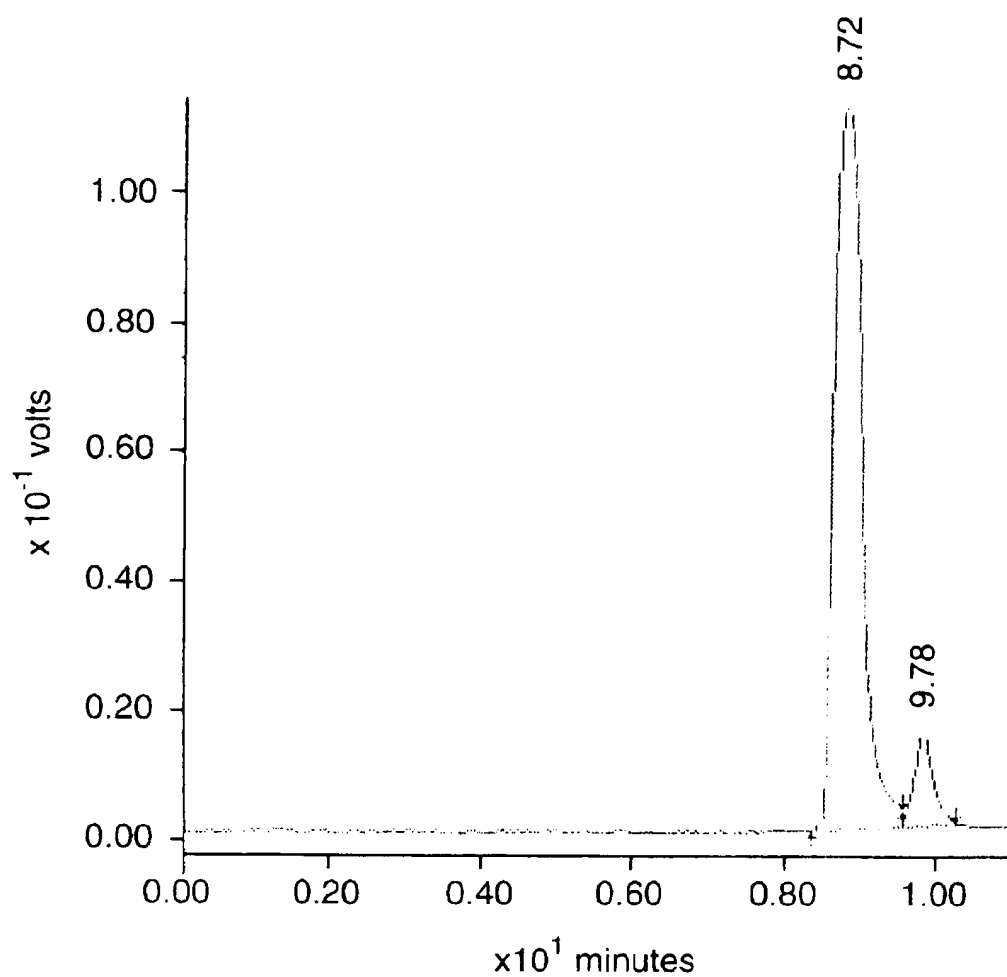
**Fig. 8**
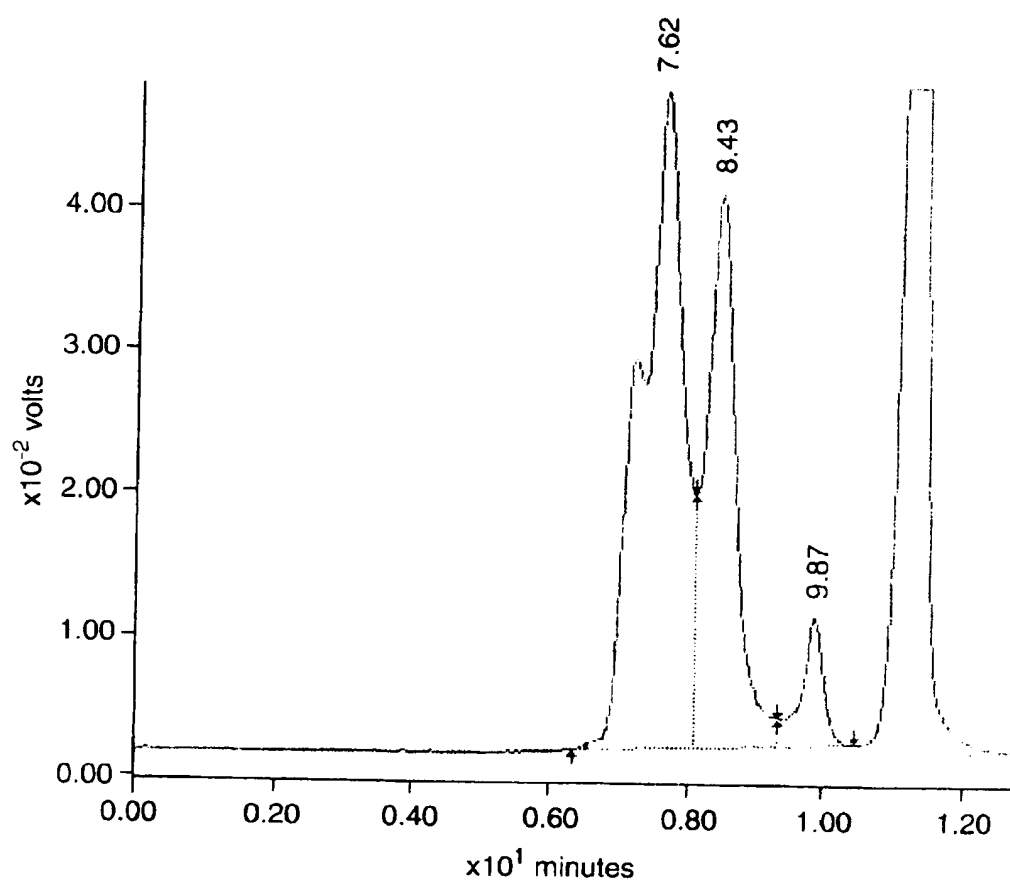
**Fig. 9**
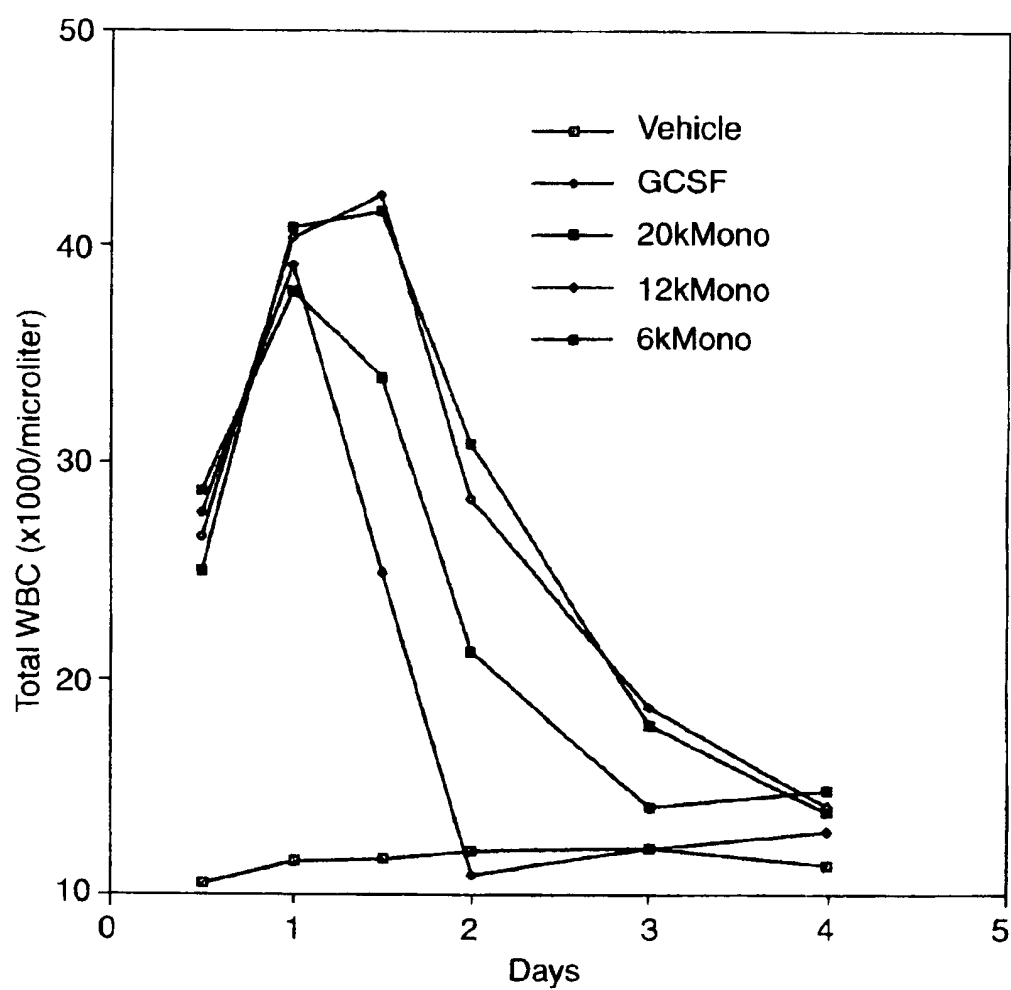
# N-TERMINALLY CHEMICALLY MODIFIED PROTEIN COMPOSITIONS AND METHODS

## FIELD OF THE INVENTION

The present invention broadly relates to the field of protein modification, and, more specifically, the attachment of water soluble polymers to proteins or analogs thereof (the term "protein" as used herein is synonymous with "polypeptide" or "peptide" unless otherwise indicated). The present invention also relates to novel methods for N-terminally modifying proteins or analogs thereof, and resultant compositions. In another aspect, the present invention relates to novel N-terminally chemically modified G-CSF compositions and related methods of preparation. The present invention also relates to chemically modified consensus interferon.

## BACKGROUND

Proteins for therapeutic use are currently available in suitable forms in adequate quantities largely as a result of the advances in recombinant DNA technologies. The availability of recombinant proteins has engendered advances in protein formulation and chemical modification. One goal of such modification is protein protection. Chemical attachment may effectively block a proteolytic enzyme from physical contact with the protein backbone itself, and thus prevent degradation. Additional advantages include, under certain circumstances, increasing the stability and circulation time of the therapeutic protein and decreasing immunogenicity. A review article describing protein modification and fusion proteins is Francis, *Focus on Growth Factors* 3: 4–10 (May 1992) (published by Mediscript, Mountview Court, Friern Barnet Lane, London N20, OLD, UK).

Polyethylene glycol ("PEG") is one such chemical moiety which has been used in the preparation of therapeutic protein products (the verb "pegylate" meaning to attach at least one PEG molecule). For example Adagen, a pegylated formulation of adenosine deaminase is approved for treating severe combined immunodeficiency disease; pegylated superoxide dismutase has been in clinical trials for treating head injury; pegylated alpha interferon has been tested in phase I clinical trials for treating hepatitis; pegylated glucocerebrosidase and pegylated hemoglobin are reported to have been in preclinical testing. The attachment of polyethylene glycol has been shown to protect against proteolysis, Sada, et al., J. Fermentation Bioengineering 71: 137–139 (1991), and methods for attachment of certain polyethylene glycol moieties are available. See U.S. Pat. No. 4,179,337, Davis et al., "Non-Immunogenic Polypeptides," issued Dec. 18, 1979; and U.S. Pat. No. 4,002,531, Royer, "Modifying enzymes with Polyethylene Glycol and Product Produced Thereby," issued Jan. 11, 1977. For a review, see Abuchowski et al., in Enzymes as Drugs. (J. S. Holcerberg and J. Roberts, eds. pp. 367–383 (1981)).

Other water soluble polymers have been used, such as copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers).

For polyethylene glycol, a variety of means have been used to attach the polyethylene glycol molecules to the protein. Generally, polyethylene glycol molecules are connected to the protein via a reactive group found on the protein. Amino groups, such as those on lysine residues or at the N-terminus, are convenient for such attachment. For

example, Royer (U.S. Pat. No. 4,002,531, above) states that reductive alkylation was used for attachment of polyethylene glycol molecules to an enzyme. EP 0 539 167, published Apr. 28, 1993, Wright, "Peg Imidates and Protein Derivates Thereof" states that peptides and organic compounds with free amino group(s) are modified with an immediate derivative of PEG or related water-soluble organic polymers. U.S. Pat. No. 4,904,584, Shaw, issued Feb. 27, 1990, relates to the modification of the number of lysine residues in proteins for the attachment of polyethylene glycol molecules via reactive amine groups.

One specific therapeutic protein which has been chemically modified is granulocyte colony stimulating factor, "G-CSF." G-CSF induces the rapid proliferation and release of neutrophilic granulocytes to the blood stream, and thereby provides therapeutic effect in fighting infection.

European patent publication EP 0 401 384, published Dec. 12, 1990, entitled, "Chemically Modified Granulocyte Colony Stimulating Factor," describes materials and methods for preparing G-CSF to which polyethylene glycol molecules are attached.

Modified G-CSF and analogs thereof are also reported in EP 0 473 268, published Mar. 4, 1992, entitled "Continuous Release Pharmaceutical Compositions Comprising a Polypeptide Covalently Conjugated To A Water Soluble Polymer," stating the use of various G-CSF and derivatives covalently conjugated to a water soluble particle polymer, such as polyethylene glycol.

A modified polypeptide having human granulocyte colony stimulating factor activity is reported in EP 0 335 423 published Oct. 4, 1989.

Another example is pegylated IL-6, EP 0 442 724, entitled, "Modified hIL-6," (see U.S. Pat. No. 5,264,209) which discloses polyethylene glycol molecules added to IL-6.

EP 0 154 316, published Sep. 11, 1985 reports reacting a lymphokine with an aldehyde of polyethylene glycol.

Many methods of attaching a polymer to a protein involve using a moiety to act as a linking group. Such moieties may, however, be antigenic. A tresyl chloride method involving no linking group is available, but this method may be difficult to use to produce therapeutic products as the use of tresyl chloride may produce toxic by-products. See Francis et al., In: Stability of protein pharmaceuticals: in vivo pathways of degradation and strategies for protein stabilization (Eds. Ahern., T. and Manning, M. C.) Plenum, N.Y., 1991) Also, Delgado et al., "Coupling of PEG to Protein By Activation With Tresyl Chloride, Applications In Immunoaffinity Cell Preparation", In: Fisher et al., eds., Separations Using Aqueous Phase Systems, Applications In Cell Biology and Biotechnology, Plenum Press, N.Y. N.Y.,1989 pp. 211–213

Chamow et al., Bioconjugate Chem. 5: 133–140 (1994) report the modification of CD4 immunoadhesin with monomethoxlypoly(ethylene glycol) aldehyde via reductive alkylation. The authors report that 50% of the CD4-Ig was McPEG-modified under conditions allowing the control over the extent of pegylation. Id. at page 137. The authors also report that the in vitro binding capability of the modified CD4-Ig (to the protein gp 120) decreased at a rate correlated to the extent of McPEGylation. Ibid. See also, Rose et al., Bioconjugate Chemistry 2: 154–159 (1991) which reports the selective attachment of the linker group carbohydrazide to the C-terminal carboxyl group of a protein substrate (insulin).

None of the methods in the general state of the art, or the art relating to particular proteins, allow for selective attach-

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ment of a water soluble polymer to the N-terminus of a protein such as G-CSF, however. Rather, the currently existing methods provide for non-selective attachment at any reactive group, whether located within the protein, such as a lysine side group, or at the N-terminus. This results in a heterogenous population. For example, for pegylated G-CSF molecules, some molecules have a different number of polyethylene glycol moieties than others. As an illustration, protein molecules with five lysine residues reacted in the above methods may result in a heterogenous mixture, some having six polyethylene glycol moieties, some five, some four, some three, some two, some one and some zero. And, among the molecules with several, the polyethylene glycol moieties may not be attached at the same location on different molecules.

This is disadvantageous when developing a therapeutic pegylated protein product. In such development, predictability of biological activity is crucial. For example, it has been shown that in the case of nonselective conjugation of superoxide dismutase with polyethylene glycol, several fractions of the modified enzyme were completely inactive (P. McGoff et al. Chem. Pharm. Bull. 36:3079–3091 (1988)). One cannot have such predictability if the therapeutic protein differs in composition from lot to lot. Some of the polyethylene glycol moieties may not be bound as stably in some locations as others, and this may result in such moieties becoming dissociated with the protein. Of course, if such moieties are randomly attached and therefore become randomly dissociated, the pharmacokinetics of the therapeutic protein cannot be precisely predictable. From a consumer's point of view, the circulation time may vary from lot to lot, and thus dosing may be inaccurate. From a producer's point of view, garnering regulatory approval for sale of the therapeutic protein may have added complexities. Additionally, none of the above methods provide for selective N-terminal chemical modification without a linking moiety (between the protein and the polymer). If a linking moiety is used, there may be disadvantages due to possible antigenicity.

Thus, there exists a need for methods allowing for selectively N-terminally chemically modified proteins and analogs thereof, including G-CSF and consensus interferon (two chemically modified proteins exemplified below). The present invention addresses this need in a number of aspects.

## SUMMARY OF THE INVENTION

The present invention relates to substantially homogenous preparations of N-terminally chemically modified proteins, and methods therefor. Unexpectedly, chemical modification at the N-terminus of G-CSF demonstrated advantages in stability which are not seen in other G-CSF species containing one chemical modification at another location on the molecule. Also unexpectedly, in the present process for making N-terminally chemically modified G-CSF, it was found that using reductive alkylation, one could provide conditions for selectively modifying the N-terminus, and this method is broadly applicable to other proteins (or analogs thereof), as well as G-CSF. Also surprisingly, using reductive alkylation, the end product—protein with an amine linkage to the water soluble polymer—was found to be far more stable than identical polymer/protein conjugate having an amide linkage. One other protein so modified (as described in a working example below) is consensus interferon. Thus, as described below in more detail, the present invention has a number of aspects relating to chemically modifying proteins (or analogs thereof) as well as specific modifications of specific proteins.

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In one aspect, the present invention relates to a substantially homogenous preparation of N-terminally chemically modified G-CSF (or analog thereof) and related methods. One working example below demonstrates that N-terminally monopegylated G-CSF more stable than other types of monopegylated C-CSF. Additionally, since the N-terminus of the G-CSF molecule is more available during reaction with polyethylene glycol, a higher proportion of the N-termini are pegylated, and therefore, this species provides processing advantages.

The present invention also relates to a type of reductive alkylation which selectively activates α-amino group of the N-terminal residue of a protein or analog thereof, thereby providing for selective attachment of a water soluble polymer moiety at the N-terminus. This provides for a substantially homogenous preparation of polymer/protein conjugate molecules as well as (if polyethylene glycol is used) a preparation of pegylated protein molecules having the polyethylene glycol moiety directly coupled to the protein moiety. This method is described below for G-CSF and for consensus interferon, and these provide for additional aspects of the present invention.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a reproduction of the chromatogram of the peaks from ion exchange chromatography of pegylated G-CSF.

FIG. 1B is an SDS-PAGE of various species of monopegylated G-CSF.

FIG. 2 is an SEC-HPLC profile of (Line A) recombinant human methionyl G-CSF standard; (Line B) SCM-PEG-GCSF reaction mix; (Line C) N-terminally pegylated G-CSF; (Line D) lysine 35 monopegylated G-CSF; (Line E) lysine 41 monopegylated G-CSF.

FIGS. 3A, 3B, and 3C are HPLC endoproteinase SV8 peptide mapping tracings of (3A) N-terminally pegylated G-CSF; (3B) lysine 35 monopegylated G-CSF; (3C) lysine 41 monopegylated G-CSF.

FIG. 4 is a bar graph illustrating a comparison of in vitro bioactivity of monopegylated G-CSF species compared to an unpegylated standard.

FIGS. 5A and 5B are graphs illustrating results of in vivo bioactivity assays of monopegylated G-CSF derivatives, with 5A illustrating the average hamster white blood cell count after a single subcutaneous injection of N-terminally pegylated G-CSF, lysine 35 monopegylated G-CSF, or lysine 41 monopegylated G-CSF, and 5B illustrating the net average white blood cell count area under the curve after a single subcutaneous injection of the various monopegylated G-CSF derivatives listed above.

FIGS. 6A, 6B, and 6C are SEC-HPLC profiles for stability studies of N-terminally pegylated G-CSF or lysine 35 monopegylated G-CSF. FIGS. 6A and 6B are the profiles for stability studies conducted at pH 6.0 at 4° C. for (6A) N-terminally monopegylated G-CSF or (6B) lysine 35 monopegylated G-CSF. FIG. 6C shows the profiles for extended stability studies at pH 6.0 and 4° C. for lysine 35 monopegylated G-CSF. Time ("T") indicates days.

FIG. 7 illustrates size exclusion HPLC analysis of the reaction mixture in the process of reductive alkylation of rh-G-CSF with methoxypolyethylene glycol aldehyde (MW 6 kDa).

FIG. 8 illustrates size exclusion HPLC analysis of the reaction mixture using N-hydroxysuccinimidyl ester of MPEG, also at MW=6kDa.

FIG. 9 illustrates the total white blood cell response after a single subcutaneous dose to mono-N terminal MPEG-GCSF conjugates prepared by reductive alkylation of rh-G-CSF with MPEG aldehydes of different molecular weights (6 kDa,12kDa and 20 kDa).

## DETAILED DESCRIPTION

The present invention relates to substantially homogenous preparations of N-terminally chemically modified proteins, and methods therefor.

In one aspect, the present invention relates to N-terminally chemically modified G-CSF compositions and methods therefor.

The present methods (for both N-terminally modified G-CSF as well as the present reductive alkylation methods) provide for a substantially homogenous mixture of monopolymer/protein conjugate. "Substantially homogenous" as used herein means that the only polymer/protein conjugate molecules observed are those having one polymer moiety. The preparation may contain unreacted (i.e., lacking polymer moiety) protein. As ascertained by peptide mapping and N-terminal sequencing, one example below provides for a preparation which is at least 90% monopolymer/protein conjugate, and at most 10% unreacted protein. Preferably, the N-terminally monopegylated material is at least 95% of the preparation (as in the working example below) and most preferably, the N-terminally monopegylated material is 99% of the preparation or more. The monopolymer/protein conjugate has biological activity. The present "substantially homogenous" N-terminally pegylated G-CSF preparations provided herein are those which are homogenous enough to display the advantages of a homogenous preparation, e.g., ease in clinical application in predictability of lot to lot pharmacokinetics.

One may choose to prepare a mixture of polymer/protein conjugate molecules, and the advantage provided herein is that one may select the proportion of monopolymer/protein conjugate to include in the mixture. Thus, if desired, one may prepare a mixture of various protein with various numbers of polymer moieties attached (i.e., di-, tri-, tetra-, etc.) and combine with the monopolymer/protein conjugate material prepared using the present methods, and have a mixture with a predetermined proportion of monopolymer/protein conjugate.

Provided below is a working example using G-CSF, which, as described above, is a therapeutic protein used to treat hematopoietic disorders. In general, G-CSF useful in the practice of this invention may be a form isolated from mammalian organisms or, alternatively, a product of chemical synthetic procedures or of prokaryotic or eukaryotic host expression of exogenous DNA sequences obtained by genomic or cDNA cloning or by DNA synthesis. Suitable prokaryotic hosts include various bacteria (e.g., E. coli); suitable eukaryotic hosts include yeast (e.g., S. cerevisiae) and mammalian cells (e.g., Chinese hamster ovary cells, monkey cells). Depending upon the host employed, the G-CSF expression product may be glycosylated with mammalian or other eukaryotic carbohydrates, or it may be non-glycosylated. The G-CSF expression product may also include an initial methionine amino acid residue (at position −1). The present invention contemplates the use of any and all such forms of G-CSF, although recombinant G-CSF, especially *E. coli* derived, is preferred, for, among other things, greatest commercial practicality.

Certain G-CSF analogs have been reported to be biologically functional, and these may also be chemically modified,

by, for example, the addition of one or more polyethylene glycol molecules. G-CSF analogs are reported in U.S. Pat. No. 4,810,643. Examples of other G-CSF analogs which have been reported to have biological activity are those set forth in AU-A-76380/91, EP 0 459 630, EP 0 272 703, EP 0 473 268 and EP 0 335 423, although no representation is made with regard to the activity of each analog reportedly disclosed. See also AU-A-10948/92, PCT US94/00913 and EP 0 243 153.

Generally, the G-CSFs and analogs thereof useful in the present invention may be ascertained by practicing the chemical modification procedures as provided herein to selectively chemically modify the N-terminal α-amino group, and testing the resultant product for the desired biological characteristic, such as the biological activity assays provided herein. Of course, if one so desires when treating non-human mammals, one may use recombinant non-human G-CSF's, such as recombinant murine, bovine, canine, etc. See PCT WO 9105798 and PCT WO 8910932, for example.

Thus, another aspect of the present invention includes N-terminally chemically modified G-CSF analog compositions. As described above, G-CSF analogs may include those having amino acid additions, deletions and/or substitutions (as compared to the G-CSF amino acid sequence set forth in Example 1, below). Those G-CSF analogs which are predicted to function when N-terminally pegylated to selectively stimulate the production of neutrophils are those with an N-terminus which is not necessary for binding to a G-CSF receptor. See Hill et al., PNAS-USA 90: 5167–5171 (1993); see also PCT US94/00913.

The polymer molecules used may be selected from among water soluble polymers. (For the reductive alkylation procedure described herein, the polymers should have a single reactive aldehyde.) The polymer selected should be water soluble so that the protein to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. For reductive alkylation, the polymer selected should have a single reactive aldehyde so that the degree of polymerization may be controlled as provided for in the present methods. The polymer may be branched or unbranched. Preferably, for therapeutic use of the end-product preparation, the polymer will be pharmaceutically acceptable. One skilled in the art will be able to select the desired polymer based on such considerations as whether the polymer/protein conjugate will be used therapeutically, and if so, the desired dosage, circulation time, resistance to proteolysis, and other considerations. For G-CSF, these may be ascertained using the assays provided herein, and one skilled in the art should select the appropriate assays for other therapeutic proteins. The water soluble polymer may be selected from the group consisting of, for example, those listed above (in the Background section), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propropylene glycol homopolymers, prolypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols and polyvinyl alcohol.

Subject to considerations for optimization as discussed below, the polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the preferred molecular weight is between about 2kDa and about 100kDa (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight). Examples 1 and 2 below involve the use of PEG 6000, which was selected for ease in purification and for providing an adequate model system. Other sizes may be used, depending

on the desired therapeutic profile (e.g., the duration of sustained release desired, the effects, if any on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of the polyethylene glycol to a therapeutic protein or analog).

One specific aspect of the present invention is N-terminally monopegylated G-CSF comprised of a polyethylene glycol moiety and a G-CSF moiety. For the present compositions, one may select from a variety of polyethylene glycol molecules (by molecular weight, branching, etc.), the proportion of polyethylene glycol molecules to G-CSF protein molecules in the reaction mix, the type of pegylation reaction to be performed, the method of obtaining the selected N-terminally pegylated G-CSF, and the type of G-CSF to be used. Further, the present compositions and methods include formulation of pharmaceutical compositions, methods of treatment and manufacture of medicaments.

The proportion of polyethylene glycol molecules to protein molecules will vary, as will their concentrations in the reaction mixture. In general, the optimum ratio (in terms of efficiency of reaction in that there is no excess unreacted protein or polymer) will be determined by the molecular weight of the polyethylene glycol selected. In addition, as one example of the present methods involves non-specific pegylation and later purification of N-terminally monopegylated species, the ratio may depend on the number of available reactive groups (typically $\alpha$ or $\ni$ amino groups) available. One working example herein involved a fairly low reaction ratio of protein:PEG molecules to obtain monopegylated material generally (1.5 PEG molecules per protein molecules).

For obtaining N-terminally pegylated G-CSF, the method for pegylation may also be selected from among various methods, as discussed above, or the present reductive alkylation as described in Example 2, below. A method involving no linking group between the polyethylene glycol moiety and the protein moiety is described in Francis et al., In: Stability of protein pharmaceuticals: in vivo pathways of degradation and strategies for protein stabilization (Eds. Ahern., T. and Manning, M. C.) Plenum, N.Y., 1991) Also, Delgado et al., "Coupling of PEG to Protein By Activation With Tresyl Chloride, Applications In Immunoaffinity Cell Preparation", In: Fisher et al., eds., Separations Using Aqueous Phase Systems, Applications In Cell Biology and Biotechnology, Plenum Press, N.Y. N.Y.,1989 pp. 211–213, involves the use of tresyl chloride, which results in no linkage group between the polyethylene glycol moiety and the protein moiety. This method may be difficult to use to produce therapeutic products as the use of tresyl chloride may produce toxic by-products. One of the present working examples involves the use of N-hydroxy succinimidyl esters of carboxymethyl methoxy polyethylene glycol. As will be discussed in more detail below, another working example involves the use of the present reductive alkylation methods.
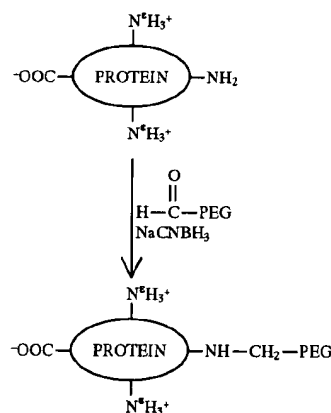
The method of obtaining the N-terminally pegylated G-CSF preparation (i.e., separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated G-CSF molecules. For example, presented below is an example where pegylated G-CSF is first separated by ion exchange chromatography to obtain material having a charge characteristic of monopegylated material (other multi-pegylated material having the same apparent charge may be present), and then the monopegylated materials are separated using size exclusion chromatography. In this way, N-terminally monopegylated G-CSF was separated

from other monopegylated species, as well as other multi-pegylated species. Other methods are reported. For example, PCT WO 90/04606, published May 3, 1990, reports a process for fractionating a mixture of PEG-protein adducts comprising partitioning the PEG/protein adducts in a PEG-containing aqueous biphasic system.

In a different aspect, the present invention provides a method for selectively obtaining an N-terminally chemically modified protein (or analog). Provided below is a method of protein modification by reductive alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl group containing polymer is achieved. The reaction is performed at pH which allows one to take advantage of the $pK_a$ differences between the $\epsilon$-amino groups of the lysine residues and that of the $\alpha$-amino group of the N-terminal residue of the protein. By such selective derivatization attachment of a water soluble polymer to a protein is controlled: the conjugation with the polymer takes place predominantly at the N-terminus of the protein and no significant modification of other reactive groups, such as the lysine side chain amino groups, occurs.

Importantly, and surprisingly, the present invention provides for a method of making a substantially homogenous preparation of monopolymer/protein conjugate molecules, in the absence of further extensive purification as is required using other chemical modification chemistries. Additionally, the product having an amine linkage is unexpectedly more stable than a product produced with an amide linkage, and this is demonstrated in the aggregation studies below. More specifically, if polyethylene glycol is used, the present invention also provides for N-terminally pegylated protein lacking possibly antigenic linkage groups, and having the polyethylene glycol moiety directly coupled to the protein moiety without toxic by-products.

The reaction may be diagrammed as follows (indicating sodium cyanohydroboride as an illustrative reducing agent):

$$N^\epsilon H_3^+$$
$$\text{-OOC} - \boxed{PROTEIN} - NH_2$$
$$N^\epsilon H_3^+$$

$$\begin{array}{c} O \\ \| \\ H - C - PEG \\ NaCNBH_3 \end{array}$$

$$N^\epsilon H_3^+$$
$$\text{-OOC} - \boxed{PROTEIN} - NH - CH_2 - PEG$$
$$N^\epsilon H_3^+$$

Thus, one aspect of the present invention is a method for preparing a polymer/protein conjugate comprised of (a) reacting a protein moiety having more than one amino group with a water soluble polymer moiety under reducing alkylation conditions, at a pH suitable to selectively activate the $\alpha$-amino group at the amino terminus of said protein moiety so that said water soluble polymer selectively attaches to said $\alpha$-amino group; and (b) obtaining the reaction product.

One may optionally, and preferably for a therapeutic product, separate the reaction products from unreacted moieties.

Another aspect of the present invention is that such reductive alkylation will provide for selective attachment of the polymer to any protein having an α-amino group at the amino terminus, and provide for a substantially homogenous preparation of monopolymer/protein conjugate. The term "monopolymer/protein conjugate" is used here to mean a composition comprised of a single polymer moiety attached to a protein moiety (also encompassed are those conjugates using protein analogs as described herein). The monopolymer/protein conjugate will have a polymer moiety located at the N-terminus, but not on amino side groups, such as those for lysine. The preparation will preferably be greater than 80% monopolymer/protein conjugate, and more preferably greater than 95% monopolymer protein conjugate.

For a substantially homogenous population of monopolymer/protein conjugate molecules, the reaction conditions are those which permit the selective attachment of the water soluble polymer moiety to the N-terminus of the desired protein. Such reaction conditions generally provide for pK_a differences between the lysine amino groups and the α-amino group at the N-terminus (the pK being the pH at which 50% of the amino groups are protonated and 50% are not). In general, for different proteins, different pH's may be used for optimally modifying the α-amino groups of the N-terminus.

The pH also affects the ratio of polymer to protein to be used. In general, if the pH is lower than the pK, a larger excess of polymer to protein will be desired (i.e., the less reactive the N-terminal α-amino group, the more polymer needed to achieve optimal conditions). If the pH is higher than the pK, the polymer:protein ratio need not be as large (i.e., more reactive groups are available, so fewer polymer molecules are needed).

Another important consideration is the molecular weight of the polymer. In general, the higher the molecular weight of the polymer, the fewer number of polymer molecules which may be attached to the protein. Similarly, branching of the polymer should be taken into account when optimizing these parameters. Generally, the higher the molecular weight (or the more branches) the higher the polymer:protein ratio.

For the present reductive alkylation, the reducing agent should be stable in aqueous solution and preferably be able to reduce only the Schiff base formed in the initial process of reductive alkylation. Preferred reducing agents may be selected from the group consisting of sodium borohydride, sodium cyanoborohydride, dimethylamine borate, trimethylamine borate and pyridine borate. Sodium cyanoborohydride was used in the working examples below.

The water soluble polymer may be of the type described above, and should have a single reactive aldehyde for coupling to the protein. For polyethylene glycol, use of PEG 6000 for coupling to G-CSF and PEG 12000 for consensus interferon are described below. It is noted, that for G-CSF, PEG 12000, 20000 and 25000 have also been used successfully in the present methods. Polyethylene glycol propionaldenhyde (see, e.g., U.S. Pat. No. 5,252,714) is advantageous for its stability in water.

As indicated above, the present methods are broadly applicable to any protein or analog thereof having an N-terminal α-amino group. For example, proteins which are the product of an exogenous DNA sequence expressed in bacteria may have, as a result of bacterially expression, an N-terminal methionyl residue with an α-amino group. As indicated above, peptides are included, as are peptidomimetics and other modified proteins. Protein analogs, such as the G-CSF analogs described above, and the non-naturally occurring consensus interferon are also suitable for the present methods.

Thus, for the present N-terminally chemically modified G-CSF, any of the G-CSF's or analogs as described herein may be used (e.g., those described supra). The working examples below use recombinant G-CSF produced in bacteria, having 174 amino acids and an extra N-terminal methionyl residue. As described herein, the chemical modification may be performed with any of the water soluble polymers described herein, and the present working examples describe the use of polyethylene glycol.

Consensus interferon is another protein used in the present working examples. Demonstrated below is the preparation of chemically modified consensus interferon using the present reductive alkylation methods for N-terminal monopegylation. Thus, other aspects of the present invention relate to these preparations. As employed herein, consensus human leukocyte interferon, referred to here as "consensus interferon," or "IFN-con", means a nonnaturally-occurring polypeptide, which predominantly includes those amino acid residues that are common to all naturally-occurring human leukocyte interferon subtype sequences and which include, at one or more of those positions where there is no amino acid common to all subtypes, an amino acid which predominantly occurs at that position and in no event includes any amino acid residue which is not extant in that position in at least one naturally-occurring subtype. IFN-con encompasses the amino acid sequences designated IFN-con$_1$, IFN-con$_2$ and IFN-con$_3$ which are disclosed in commonly owned U.S. Pat. Nos. 4,695,623 and 4,897,471, the entirety of which are hereby incorporated by reference. (U.S. Pat. Nos. 4,897,471 and 4,695,623 use the denomination "α" which is not used herein.) DNA sequences encoding IFN-con may be synthesized as described in the above-mentioned patents or other standard methods. IFN-con polypeptides are preferably the products of expression of manufactured DNA sequences, transformed or transfected into bacterial hosts, especially E. coli. That is, IFN-con is recombinant IFN-con. IFN-con is preferably produced in E. coli may be purified by procedures known to those skilled in the art and generally described in Klein et al.,J. Chromatog. 454: 205–215 (1988) for IFN-con$_1$. Purified IFN-con may comprise a mixture of isoforms, e.g., purified IFN-con$_1$ comprises a mixture of methionyl IFN-con$_1$, des-methionyl IFN-con$_1$ and des-methionyl IFN-con$_1$ with a blocked N-terminus (Klein et al., Arc. Biochem. Biophys. 276: 531–537 (1990)). Alternatively, IFN-con may comprise a specific, isolated isoform. Isoforms of IFN-con are separated from each other by techniques such as isoelectric focusing which are known to those skilled in the art.

Thus, another aspect of the present invention is a chemically modified consensus interferon wherein said consensus interferon moiety is selected from the group consisting of IFN-con$_1$, IFN-con$_2$, and IFN-con$_3$. The chemical modification is using a water soluble polymer as described herein, such as PEG, and the present reductive alkylation methods may be used for selective N-terminal chemical modification. Example 3 herein illustrates a chemically modified IFN con$_1$ comprised of an IFN con$_1$ moiety connected at the N-terminus to a polyethylene glycol moiety (PEG 12000).

In another aspect, the present methods yield pegylated proteins where the polyethylene glycol moiety is directly

attached to a protein moiety, and a separate linking group is absent and no toxic by-products are present. The examples include G-CSF and consensus interferon as described herein. For a population of pegylated G-CSF protein molecules wherein the polyethylene glycol moiety is directly attached to the G-CSF protein moiety (not necessarily a population of N-terminally pegylated G-CSF molecules), one may perform the above reductive alkylation with or without an acidic pH.

In yet another aspect of the present invention, provided are pharmaceutical compositions of the above. Such pharmaceutical compositions may be for administration for injection, or for oral, pulmonary, nasal or other forms of administration. In general, comprehended by the invention are pharmaceutical compositions comprising effective amounts of monopolymer/protein conjugate products of the invention together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (e.g., Tris-HCl, acetate, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (e.g., Tween 80, Polysorbate 80), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimersol, benzyl alcohol) and bulking substances (e.g., lactose, mannitol); incorporation of the material into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. Such compositions may influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the present N-terminally chemically modified proteins. See, e.g., Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435–1712 which are herein incorporated by reference.

In yet another aspect of the present invention, methods of treatment and manufacture of a medicament are provided. Conditions alleviated or modulated by the administration of the present polymer/G-CSF conjugates (or analogs having the hematopoietic biological properties of naturally occurring G-CSF) are typically those characterized by a reduced hematopoietic or immune function, and, more specifically, a reduced neutrophil count. Such conditions may be induced as a course of therapy for other purposes, such as chemotherapy or radiation therapy. Such conditions may result from infectious disease, such as bacterial, viral, fungal or other infectious disease. For example, sepsis results from bacterial infection. Or, such condition may be hereditary or environmentally caused, such as severe chronic neutropenia or leukemias. Age may also play a factor, as in the geriatric setting, patients may have a reduced neutrophil count or reduced neutrophil mobilization. Some of such conditions are reviewed in Filgrastim (r-met Hu G-CSF) in Clinical Practice, Morstyn, G. and T. M. Dexter, eds., Marcel Dekker, Inc., N.Y., N.Y. (1993), 351 pp. Other less-studied conditions which may be alleviated or modulated by administration of the present polymer/G-CSF conjugates may include the reduction of lipids (or cholesterol) in the blood stream, and certain cardiovascular conditions, as G-CSF may induce

production of plasminogen activators. The mode of action of G-CSF (or analogs) in these settings is not well understood at present. The addition of a water soluble polymer, such as polyethylene glycol, may provide practical patient benefits in that the sustained duration of biological activity may allow for fewer G-CSF injections per course of treatment.

Generally, conditions which may be alleviated or modulated by administration of the present polymer/consensus interferon are those to which consensus interferon is applicable and include cell proliferation disorders, viral infections, and autoimmune disorders such as multiple sclerosis. Cf., McManus Balmer, DICP, The Annals of Pharmacotherapy 24: 761–767 (1990)(Clinical use of biologic response modifiers in cancer treatment: an overview. Part I. The Interferons). Methods and compositions for the treatment of cell proliferation disorders using consensus interferon are described in PCT WO 92/06707, published Apr. 30, 1992, which is herein incorporated by reference. For example, hepatitis (A, B, C, D, E) may be treatable using the present pegylated consensus interferon molecules. The working example below demonstrates that, in vitro, chemically modified consensus interferon has 20% of the biological activity of non-chemically modified consensus interferon.

For all of the above molecules, as further studies are conducted, information will emerge regarding appropriate dosage levels for treatment of various conditions in various patients, and the ordinary skilled worker, considering the therapeutic context, age and general health of the recipient, will be able to ascertain proper dosing. Generally, for injection or infusion, dosage will be between 0.01 $\mu$g/kg body weight, (calculating the mass of the protein alone, without chemical modification), and 100 $\mu$g/kg (based on the same).

The below examples illustrate the various aspects discussed above. In Example 1, the advantages of N-terminally pegylated G-CSF are demonstrated as compared to G-CSF monopegylated at lysine-35 or lysine 41 (of the G-CSF met+174 amino acid version). Example 2 illustrates the present reductive alkylation in N-terminally pegylating G-CSF. The method provides for a substantially homogenous preparation of N-terminally pegylated G-CSF. Example 3 illustrates the present reductive alkylation in N-terminally pegylating consensus interferon.

### EXAMPLE 1

A. Preparation of Recombinant Human met-G-CSF

Recombinant human met-G-CSF (referred to as "rhG-CSF" or "r-met-hu-G-CSF" from time to time herein) was prepared as described above according to methods in the Souza patent, U.S. Pat. No. , 4,810,643, which is herein incorporated by reference. The rhG-CSF employed was an E. coli derived recombinant expression product having the amino acid sequence (encoded by the DNA sequence) shown below (Seq.ID NOs. 1 and 2):

```
ATG ACT CCA TTA GGT CCT GCT TCT TCT CTG CCG CAA AGC TTT CTG
 M   T   P   L   G   P   A   S   S   L   P   Q   S   F   L

CTG AAA TGT CTG GAA CAG GTT CGT AAA ATC CAG GGT GAC GGT GCT
 L   K   C   L   E   Q   V   R   K   I   Q   G   D   G   A

GCA CTG CAA GAA AAA CTG TGC GCT ACT TAC AAA CTG TGC CAT CCG
 A   L   Q   E   K   L   C   A   T   Y   K   L   C   H   P
```

-continued

```
GAA GAG CTG GTA CTG CTG GGT CAT TCT CTT GGG ATC CCG TGG GCT
 E   E   L   V   L   L   G   H   S   L   G   I   P   W   A

CCG CTG TCT TCT TGT CCA TCT CAA GCT CTT CAG CTG GCT GGT TGT
 P   L   S   S   C   P   S   Q   A   L   Q   L   A   G   C

CTG TCT CAA CTG CAT TCT GGT CTG TTC CTG TAT CAG GGT CTT CTG
 L   S   Q   L   H   S   G   L   F   L   Y   Q   G   L   L

CAA GCT CTG GAA GGT ATC TCT CCG GAA CTG GGT CCG ACT CTG GAC
 Q   A   L   E   G   I   S   P   E   L   G   P   T   L   D

ACT CTG CAG .CTA GAT GTA GCT GAC TTT GCT ACT ACT ATT TGG CAA
 T   L   Q   L   D   V   A   D   F   A   T   T   I   W   Q

CAG ATG GAA GAG CTC GGT ATG GCA CCA GCT CTG CAA CCG ACT CAA
 Q   M   E   E   L   G   M   A   P   A   L   Q   P   T   Q

GGT GCT ATG CCG GCA TTC GCT TCT GCA TTC CAG CGT CGT GCA GGA
 G   A   M   P   A   F   A   S   A   F   Q   R   R   A   G

GGT GTA CTG GTT GCT TCT CAT CTG CAA TCT TTC CTG GAA GTA TCT
 G   V   L   V   A   S   H   L   Q   S   F   L   E   V   S

TAC CGT GTT CTG CGT CAT CTG GCT CAG CCG TAA TAG
 Y   R   V   L   R   H   L   A   Q   P   *   *
```

(This was also the non-pegylated composition used for the control animals.) Alternatively one may use purchased NEUPOCOEN® for the following pegylation procedures (the package insert for which is herein incorporated by reference).

B. Preparation of Pegylated G-CSF

A 10 mg/ml solution of the above rh-G-CSF, in 100 mM Bicine pH 8.0, was added to solid SCM-MPEG (N-hydroxy succinimidyl esters of carboxymethyl methoxy polyethylene glycol) (Union Carbide) with an average molecular weight of 6000 Daltons. This gave a 1.5 molar excess of SCM-MPEG to rh-G-CSF. After one hour with gentle stirring, the mixture was diluted to 2 mg/ml with sterile water, and the pH was adjusted to 4.0 with dilute HCl. The reaction was carried out at room temperature. At this stage, the reaction mixture consisted mainly of three forms of mono-pegylated rh-G-CSF, some di-pegylated rh-G-CSF, unmodified rh-G-CSF and reaction bi-product (N-hydroxy succinimide).

C. Preparation of N-terminally Pegylated rh-G-CSF

The three forms of monopegylated rh-G-CSF were separated from each other using ion exchange chromatography. The reaction mixture was loaded (1 mg protein/ml resin) onto a Pharmacia S SEPHAROSE FF column (Pharmacia XK50/30 reservoir, bed volume of 440 ml) equilibrated in buffer A (20 mM sodium acetate, pH 4.0). The column was washed with 3 column volumes of buffer A. The protein was eluted using a linear gradient from 0–23% buffer B (20 mM sodium acetate, pH 4.0, 1M NaCl) in 15 column volumes. The column was then washed with one column volume of 100% buffer B and reequilibrated with 3 column volumes of buffer A. The flow rate for the entire run was maintained at 8 ml/min. The eluent was monitored at 280 nm and 5 ml fractions were collected. Fractions containing the individual monopegylated species were pooled according to FIG. 1A. These pools were concentrated with a 350 mL Amicon stirred cell using a YM10 76 mm membrane.

Pooled fractions from the ion exchange chromatography were subjected to size exclusion chromatography to separate di-pegylated species from monopegylated species. Typically, 5–10 mg in 2–5 ml of solution were loaded onto a 120 ml Pharmacia SUPERDEX 75 HR 16/60 column equilibrated with 20 mM sodium acetate pH 4.0. The column was run at 1.5 ml/min for 100 min. Two ml fractions were collected. The protein content of the eluent was monitored at 280 nm. Fractions from separated peaks were pooled and subjected to analysis. The table below compares the proportional yields for each peak.

### TABLE 1

| Relative Yields and Site of Modification | | |
| --- | --- | --- |
| Site of Modification | Figure 1A Reference | Relative Yields |
| N-Terminus | Peak 1A | 3 |
| Lysine-35 | Peak 2A | 2 |
| Lysine-41 | Peak 3A | 1 |

Under these conditions, the lysines at positions 17 and 24 probably were not significantly pegylated.

D. Characterization

Five analyses were done to characterize each sample: (1) SDS-Page (FIG. 1B), (2) Size exclusion chromatography HPLC ("SEC HPLC")(FIG. 2), (3) peptide mapping analysis (FIGS. 3A, 3B, and 3C), (4) in vitro G-CSF bioassay (FIG. 4), and (5) in vivo testing in hamster (FIGS. 5A and 5B).

With regard to the composition of each sample, results demonstrate that, of the N-terminally monopegylated G-CSF, the samples showed a greater than 95% N-terminally pegylated composition, with the remainder probably being unpegylated material (although the remainder of the samples is lower than the detection limit of the assay). With regard to the percent monopegylated for each of the three types of monopegylated material (N-terminal, pegylated at lysine 35, and pegylated at lysine 41), the N-terminal and the lysine 41 demonstrated greater than 97% monopegylated, and the lysine 35 pegylated material being somewhat lower, probably due to the instability of the molecule in the assay conditions. To summarize, the following results were obtained:

TABLE 2

Percent Composition of
N-terminally pegylated G-CSF

| | Non-Reduced SDS PAGE | SEC HPLC | N-Terminal Sequencing* |
|---|---|---|---|
| Mono-pegylated G-CSF | 97.44 | 99.43 | 96.6 |
| Unmodified G-CSF | 2.56 | 0.57 | 3.4 |

*The N-terminal sequencing, as discussed infra is not here considered quantitative, as there may have been artifactual separation of the polyethylene glycol molecule from the N-terminus of the protein during the sequencing process.

TABLE 3

Percent Monopegylated for Three Species

| | N-terminal PEG-GCSF (RI/UV = .96)* | LYS35 PEG-GCSF** (RI/UV = .72) | LYS41 PEG-GCSF (RI/UV = 1.12) |
|---|---|---|---|
| Non-reduced SDS-PAGE | 97.44 | 77.41 | 100.00 |
| SEC HPLC | 99.43 | 93.38 | 99.96 |

*RI/UV refers to the Index of Refraction/Ultraviolet light absorbance ratio, and is used to estimate the number of polyethylene glycol molecules per molecule of protein. It is calculated from the SEC HPLC data using an Index of Refraction for polyethylene glycol and an ultraviolet absorbance for protein.
**Note that this species is unstable under the assay conditions used.

## METHODS

### 1. SDS-PAGE.

SDS-PAGE was carried out in a non-reduced 4–20% ISS Daiichi Pure Chemicals, Co., Tokyo, Japan minigel using a Coomassie Brillant Blue R-250 stain. The gel was scanned using a molecular Dynamics Densitometer with Image Quant. Results: Results are presented in FIG. 1B. Lane number 1 (from the left hand side) included molecular weight protein standards (Novex Mark 12 Molecular Weight Standards). Lane 2 contains 3 μg rh-G-CSF standard. Lane 3 contains the SCM-PEG-GCSF reaction mix, with 10 μg loaded. Lane 4 contains N-terminally monopegylated G-CSF, with 10 μg loaded. Lane 5 contains 10 μg of monopegylated G-CSF with the pegylation site at the lysine found at the 35th residue from the N-terminal methionine. Lane 6 contains 10 μg of monopegylated G-CSF with the pegylation site at the lysine found at the 41st residue from the N-terminal methionine. As can be seen, Lane 3, containing the N-terminally monopegylated material, shows a single band

### 2. Size Exclusion Chromatography-High Pressure Liquid Chromatography.

SEC-HPLC was carried out using a Waters HPLC system with a BIOSEP SEC 3000 column, using 100 mM sodium phosphate, pH 6.9, 1ml/min for 20 minutes. The signal was monitored at 280 nm. Results: As can be seen from FIG. 2, line "C," containing the N-terminally monopegylated rh-G-CSF contains a single peak, as do lines "D" (Lys-35 monop-egylated material) and "E" (Lys-41 monopegylated material). This indicates substantial purity among the separated fractions of monopegylated G-CSF.

### 3. Peptide mapping.

The following methods were used. Three samples, called "Mono-PEG 1", "Mono-PEG-2", and "Mono-PEG-3", were analyzed. (a) Reductive alkylation. 500 μg aliquots of mono-

PEG G-CSF were speed vac dried and reconstituted to a concentration of 1 mg in 950 μl in 0.3M Tris-HCl containing 6M Guanidinum HCl and 1 mM EDTA pH 8.4. Samples were then S-carboxymethylated by adding iodoacetic acid and incubated at 37° C. for 20 minutes. Samples were then desalted using SEPHADEX G+25 Quick Spin Protein Columns and buffer exchanged. After desalting and buffer exchange, sample concentration was adjusted to 0.5 mg/ml using additional buffer. (b) Endoproteinase SV8 digestion. Samples were digested with SV8 (enzyme to substrate ratio of 1:25) at 25° C. for 26 hours.(c) HPLC peptide mapping. Protein digests were injected onto a Vydac C4 column (4.6×250 mm, 5 μ particle size, 300 Å pore size) and peptides were mapped by HPLC using a linear gradient of acetonitrile in 0.1% TFA. Peptides were manually collected and dried in a Speed Vac for sequence analysis. Results: As compared to a reference standard, (i) (FIG. 3A) for "Mono-PEG-1", (the N-terminally mono-pegylated material), a peak at 57.3 minutes diminished and a new peak appeared at 77.5 minutes; (ii) (FIG. 3B) for "Mono-PEG-2", (the lysine 35 pegylated material), there was a decrease in peak height for a peptide with a retention time of 30.3 minutes, and a new peak eluted at 66.3 minutes; (iii) (FIG. 3C) for "Mono-PEG-3" (the lysine 41 pegylated material), the peak at retention time of 30.3 minutes was missing, and a new peak appeared at 66.4 minutes. These peptides were the only significant differences in the sample maps. There were some small incomplete cleavages seen on either side of the peptide at 86.1 minutes due to minor digestion differences. (d) N-terminal sequence analysis. Each of the "new" peptides in the above maps were N-terminally sequenced for identification. The dried peptides were reconstituted in 0.1% TFA and sequenced on an ABI protein sequencer. For "Mono-PEG-1" (the N-terminally pegylated material), 60% of the "new" peak (at 77.5 minutes) was sequenced for 10 cycles. The initial yield was less than 5%, indicating that the N-terminal methionyl residue is blocked by a polyethylene glycol molecule. It is noted that this initial peptide should have resulted in a zero initial yield, and the <5% yield observed may be from detachment of the polyethylene glycol from the N-terminal methionyl during sequence analysis. The sequence detected was that of the N-terminal peptide, M-T-P-L-G-P-A-S-S. For "Mono-PEG-2", (the lysine 35 pegylated material), 80% of the total peak volume was collected for the peak at 66.3 minutes, and was sequenced for 9 cycles. The recovery of lysine 35 was significantly low, indicating pegylation at position 35. The recovery of lysine 41 was consistent with the other residue, indicating no modification of this position. The peptide at 30.3 minutes decreased in peak height compared to the corresponding peak in the standard reference map. The peptide at 30.3 minutes is only 57.5% of the peak area of the corresponding peptide. The sequence detected for this species was K-L-C-A-T-Y-K-L. For "Mono-PEG-3", the lysine 41 material, 80% of the total peak volume collected for the peptide eluting at 66.4 minutes was sequenced for 9 cycles. The sequence detected was K-L-C-A-T-Y-K-L, and contained lysine residues 35 and 41. The recovery of lysine 35 was consistent with other residue recoveries. The recovery of lysine 41 was significantly lower indicating pegylation at position 41. Results: "Mono-PEG-1" is N-terminally monopegylated material; "Mono-PEG-2" is lysine 35 partially pegylated; and "Mono-PEG-3" is lysine 41 pegylated material. By comparing both the reference standard (non-pegylated G-CSF) and GCSF monopegylated 1, 2, and 3 peptide maps, it was found that both the "Mono-PEG-2" (lysine 35) and "Mono-PEG-3" (lysine 41) maps exhibit

slightly diminished peak heights for the N-terminal peptide. This indicates that the lysine 35 and lysine 41 material contains a small amount of N-terminally pegylated material or that the N-terminal methionine has a small percentage of pegylation.

4. In vitro activity.

The material was active. FIG. 4 illustrates the results of in vitro assays. As can be seen, the N-terminally monopegylated material had 68% of the activity of non-modified rhG-CSF. Methods: The G-CSF in vitro bioassay is a mitogenic assay utilizing a G-CSF dependent clone of murine 32D cells. Cells were maintained in Iscoves medium containing 5% FBS and 20 ng/ml rhG-CSF. Prior to sample addition, cells were prepared by rinsing twice with growth medium lacking rhG-CSF. An extended twelve point rhG-CSF standard curve was prepared, ranging from 48 to 0.5ng/ml (equivalent to 4800 to 50 IU/ml). Four dilutions, estimated to fall within the linear portion of the standard curve, (1000 to 3000 IU/ml), were prepared for each sample and run in triplicate. Because of their apparent lower activity in vitro, the pegylated rhG-CSF samples were diluted approximately 4–10 times less. A volume of 40 $\mu$l of each dilution of sample or standard is added to appropriate wells of a 96 well microtiter plate containing 10,000 cells/well. After forty-eight hours at 37° C. and 5.5% $CO_2$, 0.5$\mu$mCi of methyl-$^3$H-thymidine was added to each well. Eighteen hours later, the plates were then harvested and counted. A dose response curve (log rhG-CSF concentration vs. CPM-background) was generated and linear regression analysis of points which fall in the linear portion of the standard curve was performed. Concentrations of unknown test samples were determined using the resulting linear equation and correction for the dilution factor. Results: Results are presented in FIG. 4. As can be seen, of the three monopegylated species, N-terminally monopegylated G-CSF demonstrates the highest in vitro biological activity.

5. In vivo activity.

In vivo testing confirmed the activity of the N-terminally pegylated material. The in vivo testing was carried out by dosing male golden hamsters with a 0.1 mg/kg of sample, using a single subcutaneous injection. Four animals were subjected to terminal bleeds per group per time point. Serum samples were subject to a complete blood count on the same day that the samples were collected. The average white blood cell counts were calculated. As can be seen in FIGS. 5A and 5B, the response from each material peaks after one day following a single subcutaneous injection of 0.1 mg/kg. Two of the monopegylated materials, (N-terminal and Lys-35) showed prolonged responses, while the response for the protein pegylated at lysine-41 showed no increase in in vivo activity over unmodified rhG-CSF (indeed it shows less, FIG. 5B). These results illustrate that attaching a single polyethylene glycol molecule can dramatically alter the therapeutic profile of a protein and that the benefit of pegylating a protein can be dependent upon the site of modification. (The net average WBC area under the curve after the single subcutaneous injection (calculated according to CRC Standard Mathematical Tables, 26th Ed. (Beyer, W. H., Ed.) CRC Press Inc., Boca Raton, Fla. 1981. p. 125) was similar for the Lys-35 and N-terminal monopegylated species.)

E. Stability Studies

In addition, stability studies were performed on the N-terminal and Lys-35 monopegylated species as prepared above. (The Lys-41 material was not used as it demonstrated no additional activity beyond unmodified G-CSF). These studies demonstrate that the N-terminally pegylated G-CSF

is unexpectedly more stable upon storage than the other form of monopegylated G-CSF, monopegylated lysine 35. Stability was assessed in terms of breakdown of product, as visualized using SEC-HPLC. Methods: N-terminally pegylated G-CSF and lysine-35 monopegylated G-CSF were studied in two pH levels, pH 4.0 and pH 6.0 at 4° C., each for up to 16 days. Elevating the pH to 6.0 provides an environment for accelerated stability assays. For the pH 6.0 samples, N-terminal monopegylated G-CSF and Lysine 35 monopegylated G-CSF as prepared above were placed in a buffer containing 20 mM sodium phosphate, 5 mM sodium acetate, 2.5 % mannitol, 0.005 % TWEEN-80, pH 6.0 at a final protein concentration of 0.25 mg/ml. One ml aliquots were stored in 3 ml sterile injection vials. Vials of each was stored at 4° C. and 29° C. for up to 16 days. Stability was assessed by SEC-HPLC tracings. If the later measurements stayed the same (as ascertained by visual inspection) as the initial (Time=0) measurements, the sample was considered to be stable for that length of time. Results: Results are illustrated in FIGS. 6A–6C. (a) Comparison at pH 6.0 at 4° C. FIG. 6A shows the 4° C. SEC-HPLC profiles for N-terminally monopegylated G-CSF at pH 6 over time and FIG. 6B shows the 4° C. SEC-HPLC profiles for lysine-35 monopegylated G-CSF at pH 6 over time. One interpretation is that the Lys-35 material is breaking down to a material with a molecular weight similar to that of unmodified G-CSF. (b) Extended duration at pH 4.0 at 4° C. PH 4.0 and 4° C. provides something of a control illustrating relatively stable conditions in that the N-terminal species shows no degradation. For the Lys 35 species, the break down of the material is still occurring, but at a much slower rate. (c) Comparison at pH 6.0 at 4° C. FIGS. 6C illustrates the SEC-HPLC profiles for the monopegylated G-CSF's under these conditions, under extended time periods. As can be seen, at pH 6.0 and 4° C., the lysine-35 material exhibits no increase in depegylation at day 16 or day 35 beyond what was seen for day 6 (FIG. 6B). This indicates that depegylation (instability) does not change, under those conditions, beyond day 6.

EXAMPLE 2

This example demonstrates a method of preparing a substantially homogenous population of monopegylated G-CSF using reductive alkylation, and characterization of this population. Recombinant G-CSF as described in the above example was used. As can be seen, not only do the present methods provide advantages in terms of yield of N-terminally chemically modified material, but also, the amine linkages of the present reductive alkylation process produce substantially more stable products as demonstrated by a large difference in the degree of aggregation upon storage.

A. Preparation of the mono-methoxypolyethylene glycol-GCSF conjugates with the site of attachment at the N-terminal $\alpha$-amino residue.

To a cooled (4° C),stirred solution ofrhG-CSF (1 ml, 5 mg/ml as described in the Example above) in 100 mM sodium phosphate, pH 5, containing 20 mM NaCNBH3, was added a 5-fold molar excess of methoxypolyethylene glycol aldehyde (MPEG)(average molecular weight, 6 kDa). The stirring of the reaction mixture was continued at the same temperature.

The extent of the protein modification during the course of the reaction was monitored by SEC HPLC using BIO-SIL SEC 250-5 column (BIO-RAD) eluted with 0.05M $NaH_2PO_4$,0.05M $Na_2HPO_4$,0.15M NaCl, 0.01M $NaN_3$, pH 6.8 at 1 ml/min.

After 10 hours the SEC HPLC analysis indicated that 92% of the protein has been converted to the mono-MPEG-GCSF derivative. This can be seen in FIG. 7, which is a recording of the protein concentration (as determined by absorbance at $A_{280}$) and shows the peak eluting at 8.72 minutes as monopegylated G-CSF, and a minor peak of unreacted G-CSF eluting at 9.78 minutes.

As a comparison, FIG. 8 shows the peaks obtained when using N-hydroxysuccinimidyl ester of MPEG. The molecular weight was also 6 kDa. As can be seen, the mixture obtained from this reaction was: tri-MPEG-GCSF conjugated (shoulder at approximately 7.25 minutes), di-MPEG-GCSF conjugate (peak at 7.62 minutes), mono-MPEG-GCSF conjugate (peak at 8.43 minutes) and unreacted G-CSF (peak at 9.87 minutes).

At this 10 hour time point, where 92% of the protein had been converted to monopegylated material, the pH of the reaction mixture was adjusted to pH 4 with 100 mM HCl and the reaction mixture was diluted 5 times with 1 mM HCl.

The mono-MPEG-GCSF derivative was purified by ion exchange chromatography using HiLoad 16/10 S SEPHAROSE HP column (Pharmacia) equilibrated with 20 mM sodium acetate buffer, pH 4. The reaction mixture was loaded on the column at a flow rate of 1 ml/min and the unreacted MPEG aldehyde eluted with three column volumes of the same buffer. Then a linear 400 minute gradient from 0% to 45% 20 mM sodium acetate, pH 4, containing 1M NaCl was used to the elute the protein-polymer conjugate at 4° C.

Fractions containing the mono-MPEG-GCSF derivative were pooled, concentrated and sterile filtered.

Various mono-MPEG-GCSF conjugates obtained by modifying rh-G-CSF with MPEG aldehydes of different average molecular weight (12, 20 and 25 kDa) were prepared in a similar manner.

B. Analysis of Monopeaylated G-CSF

1. Molecular Weight

The molecular weight at the monopegylated conjugates was determined by SDS-PAGE, gel filtration, matrix assisted laser desorption mass spectrometry, and equilibrium centrifugation. These results are presented in Table 4, below.

TABLE 4

| | Molecular Weights of N-terminally Alkylated Mono-MPEG-GCSF Conjugates | | | |
|---|---|---|---|---|
| Conjugate | MW estimated | MW gel filtration | MW mass spectometry | MW ultra-centrifugation |
| MPEG-(6kDa)-GCSF | 24800 | 53024 | 24737 | 25548 |
| MPEG-(12kDa)-GCSF | 30800 | 124343 | 30703 | 29711 |
| MPEG-(20kDa)-GCSF | 38800 | 221876 | 38577 | 38196 |
| MPEG-(25kDa)-GCSF | 43800 | 333266 | N/D | |

The structure of the prepared N-terminal mono-MPEG-GCSF conjugates was confirmed using the methods of N-terminal protein sequencing and peptide mapping. Cyanogen bromide cleavage of the N-terminal methionyl residue resulted in removal of the polyethylene glycol.

2. Biological Activity

The in vitro biological activity of the pegylated MPEG-GCSF conjugates was determined by measuring the stimulated uptake of $^3H$ thymidine into mouse bone marrow cells.

The in vivo biological activity was determined by subcutaneous injection to hamsters MPEG-GCSF conjugates or rhG-CSF (at 100 mg/kg) and measuring total white blood cell count. Bioactivity as compared to non-derivatized G-CSF was calculated as the area under the WBC/time curve after subtracting the vehicle control curve. Relative bioactivity of the MPEG-GCSF derivatives was expressed as the percentage bioactivity compared to unmodified G-CSF.

This is illustrated in FIG. 9, which is a graph illustrating the total white blood cell response to mono-N-terminal MPEG-GCSF conjugates prepared by reductive alkylation of rhG-CF with MPEG aldehydes of different molecular weights (6 kDa, 12 kDa, and 20 kDa). As can be seen, all monopegylated molecules elicited a response. The higher the molecular weight of the polyethylene glycol moiety used, the higher the white blood cell count achieved, except the 12 kDa achieved a slightly higher count than did the 20 kDa version at day 2.

3. Stability Studies N-terminally pegylated G-CSF's prepared by the two different chemistries (amide vs. amine of the reductive alkylation here) were compared for the degree of aggregation. Unexpectedly, N-terminally pegylated G-CSF using the amine chemistry was found to be substantially more stable than N-terminally pegylated G-CSF with an amide linkage (NHS chemistry as described in Example 1).

Methods: Both N-terminally pegylated G-CSF samples were in 10 mM NaOac pH4.0 with 5% sorbitol, at a concentration of 1 mg protein/ml. The G-CSF's were pegylated with PEG 6000 for each. The amide-linked conjugate was prepared as in Example 1, and the amine linked conjugate was prepared as in Example 2. Six samples of each were stored for eight weeks at 45° C. At the end of eight weeks, the degree of aggregation was determined using size exclusion chromatography and ion exchange chromatography.

Results: The results demonstrate that the present reductive alkylation methodis advantageous over aceylation because, surprisingly, it produces a material with far fewer aggregates after 8 weeks at elevated temperatures. The table below shows the percent of non-aggregated material ("main peak" material) for both materials using size exclusion chromatography (SEC) or ion exchange (IE):

TABLE 5

| Sample:8 wkg, 45° C. | % Main Peak SEC/IE |
|---|---|
| Amine | 82%/84% |
| Amide | 37%/65%* |

*This is relatively high because ion exchange does not allow for full analysis of the aggregation.

EXAMPLE 3

This example demonstrates chemically modified consensus interferon. More specifically, this example demonstrates a method of preparing a substantially homogenous population of monopegylated IFN-con$_1$, and characterization of this population.

It should be noted that while the present example uses IFN-con$_1$, any of the consensus interferons as set forth above may be chemically modified. Such chemical modification may be with any of the water soluble polymers as listed above, although PEG is used here. For pegylation, PEG 12000 is used here, although any water soluble PEG species may be used (PEG 12000 was selected for ease in handling

and convenience). Again, a variety of means for chemical modification are available (such as acetylation) but, for selective N-terminal chemical modification, such as N-terminal pegylation, the present reductive alkylation method as described in this example is preferred.

A. Preparation of Consensus Interferon

IFN-αcon₁ (here referred to as IFN-con₁) as described in FIG. 2 of U.S. Pat. No. 4,695,623, which is incorporated by reference in its entirety, was used for the preparation of monopegylated consensus interferon. The IFN-con₁ was produced by expression of exogenous DNA in bacteria, and contained a methionyl residue at the N-terminus.

B. Pegylation of Consensus Interferon

To a cooled (4° C.), stirred solution of IFN-con₁ (3.45 mg/ml, containing 35.25% of the N-terminally blocked form) in 100 mM sodium phosphate, pH 4.0, containing 20 mM NaCNBH₃ was added a 8-fold molar excess of methoxypolyethylene glycol aldehyde (MPEG)(average molecular weight 12 kDa).

The extent of the protein modification during the course of the reaction was monitored by reverse phase HPLC using a polymer-based poly(styrene/divinylbenzene) column, such as PLRP-S (PL Separation Sciences Polymer Laboratories).

After 10 hours the reverse phase HPLC analysis indicated that 80% of the protein with unblocked α-amino group at the N-terminus has been converted to the MPEG-IFN-con₁ derivative.

At the 10 hour time point, the reaction mixture was diluted 5 times with water and the mono-MPEG-IFN-Con₁ derivative was purified by ion exchange chromatography using HiLoad 16/10 S SEPHAROSE HP column (Pharmacia) equilibrated with 20 mM sodium acetate buffer, pH 4.0. The reaction mixture was loaded on the column at a flow rate of 1 ml/min and the unreacted MPEG aldehyde eluted with three column volumes of the same buffer. Then a linear 420 minute gradient from 0% to 75% of 20 mM sodium acetate, pH 4.0, containing 1M NaCl was used to the elute the protein-polymer conjugate at 4° C.

Fractions containing the mono-MPEG-IFN-Con₁ derivative were pooled, concentrated and sterile filtered.

C. Analysis of Monopeaylated Consensus Interferon

1. Homogeneity

The homogeneity of the purified mono-MPEG-IFN-Con₁ conjugates was determined by SDS-PAGE using 10–20% or 4–20% precast gradient gels (Integrated Separation Systems). The gels showed a main band at MW 35 kDa.

To characterize the effective size (hydrodynamic radius) of each mono-MPEG-IFN-con₁ species a SUPEROSE 6 HR 10/30 (Pharmacia) gel filtration column was used. Proteins were detected by UV absorbance at 280 nm. The BIO-RAD gel filtration standards served as globular protein molecular weight markers.

The structure of the purified N-terminal mono-MPEG-IFN-con₁ conjugates was confirmed using the methods of N-terminal protein sequencing and peptide mapping.

It is noted that this IFN-con₁ preparation contained some N-terminally blocked material, and this material was not pegylated. The material which was pegylated, however, was monopegylated at the N-terminus. Thus, in this type of situation, one may wish to use other means to separate the blocked from the unblocked material, such as ion exchange or size exclusion chromatography.

2. Biological Activity

The in vitro biological activity of the mono-MPEG-IFN Con₁ conjugates was determined by measuring their antiviral bioactivity. The in vitro biological activity of the mono-MPEG-IFN-Con₁ conjugates was determined by measuring their antiviral bioactivity in human (HeLa) cells.

It was found that the mono-MPEG (12 kDa)-IFN-Con₁ conjugate shows 20% in vitro bioactivity (in U/mg of protein) when compared to the unmodified species. As noted above for pegylated G-CSF, the in vitro assays, while useful to demonstrate biological activity, may show a rather low level of activity for chemically modified proteins because of characteristic sustained release. The in vivo biological activity may be higher than the in vitro biological activity.

D. Chemically modified consensus interferon with the N-terminally blocked molecules removed

The present reductive alkylation was also performed on the above IFN-con₁ which had the portion of N-terminally blocked molecules pre-removed. Both PEG 12000 and PEG 20000 were used in the reductive alkylation method as described above.

The molecular apparent molecular weights were as follow:

| Conjugate | Apparent MW by Gel Filtration | Apparent MW by SDS-PAGE |
|---|---|---|
| monoMPEG(12kDa) IFN-con₁ | 104.0 kDa | 35.6 kDa |
| monoMPEG(20kDa) IFN-con₁ | 175.1 kDa | 55.4 kDa |

Analysis of the IFN-con₁ 20 kDa PEG conjugate using FPLC ion exchange chromatography resulted in three peaks:

MonoMPEG-IFN-con₁: 66% of the total area (eluting at 265.93 ml)

Protein aggregate and oligo MPEG-IFN-con₁ conjugate: 24% of the total area (eluting at 238.42 ml); and

Unreacted IFN-con₁: 10% of the total area (eluting at 328.77 ml).

The conditions were not further optimized. One may further separate the monopegylated material using chromatographic or other methods.

While the present invention has been described in terms of preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations which come within the scope of the invention as claimed.

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SEQUENCE LISTING

( 1 ) GENERAL INFORMATION:

( 1 1 1 ) NUMBER OF SEQUENCES: 2

( 2 ) INFORMATION FOR SEQ ID NO:1:

( i ) SEQUENCE CHARACTERISTICS:
    ( A ) LENGTH: 531 base pairs
    ( B ) TYPE: nucleic acid
    ( C ) STRANDEDNESS: single
    ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: cDNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
ATGACTCCAT  TAGGTCCTGC  TTCTTCTCTG  CCGCAAAGCT  TTCTGCTGAA  ATGTCTGGAA    60

CAGGTTCGTA  AAATCCAGGG  TGACGGTGCT  GCACTGCAAG  AAAAACTGTG  CGCTACTTAC   120

AAACTGTGCC  ATCCGGAAGA  GCTGGTACTG  CTGGGTCATT  CTCTTGGGAT  CCCGTGGGCT   180

CCGCTGTCTT  CTTGTCCATC  TCAAGCTCTT  CAGCTGGCTG  GTTGTCTGTC  TCAACTGCAT   240

TCTGGTCTGT  TCCTGTATCA  GGGTCTTCTG  CAAGCTCTGG  AAGGTATCTC  TCCGGAACTG   300

GGTCCGACTC  TGGACACTCT  GCAGCTAGAT  GTAGCTGACT  TTGCTACTAC  TATTTGGCAA   360

CAGATGGAAG  AGCTCGGTAT  GGCACCAGCT  CTGCAACCGA  CTCAAGGTGC  TATGCCGGCA   420

TTCGCTTCTG  CATTCCAGCG  TCGTGCAGGA  GGTGTACTGG  TTGCTTCTCA  TCTGCAATCT   480

TTCCTGGAAG  TATCTTACCG  TGTTCTGCGT  CATCTGGCTC  AGCCGTAATA  G           531
```

( 2 ) INFORMATION FOR SEQ ID NO:2:

    ( i ) SEQUENCE CHARACTERISTICS:
        ( A ) LENGTH: 175 amino acids
        ( B ) TYPE: amino acid
        ( C ) STRANDEDNESS: single
        ( D ) TOPOLOGY: linear

    ( i i ) MOLECULE TYPE: protein

    ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met  Thr  Pro  Leu  Gly  Pro  Ala  Ser  Ser  Leu  Pro  Gln  Ser  Phe  Leu  Leu
 1              5                        10                       15

Lys  Cys  Leu  Glu  Gln  Val  Arg  Lys  Ile  Gln  Gly  Asp  Gly  Ala  Ala  Leu
               20                       25                  30

Gln  Glu  Lys  Leu  Cys  Ala  Thr  Tyr  Lys  Leu  Cys  His  Pro  Glu  Glu  Leu
               35                  40                  45

Val  Leu  Leu  Gly  His  Ser  Leu  Gly  Ile  Pro  Trp  Ala  Pro  Leu  Ser  Ser
          50                  55                  60

Cys  Pro  Ser  Gln  Ala  Leu  Gln  Leu  Ala  Gly  Cys  Leu  Ser  Gln  Leu  His
65                       70                  75                       80

Ser  Gly  Leu  Phe  Leu  Tyr  Gln  Gly  Leu  Leu  Gln  Ala  Leu  Glu  Gly  Ile
               85                       90                  95

Ser  Pro  Glu  Leu  Gly  Pro  Thr  Leu  Asp  Thr  Leu  Gln  Leu  Asp  Val  Ala
               100                      105                      110

Asp  Phe  Ala  Thr  Thr  Ile  Trp  Gln  Gln  Met  Glu  Glu  Leu  Gly  Met  Ala
          115                      120                      125

Pro  Ala  Leu  Gln  Pro  Thr  Gln  Gly  Ala  Met  Pro  Ala  Phe  Ala  Ser  Ala
          130                      135                      140

Phe  Gln  Arg  Arg  Ala  Gly  Gly  Val  Leu  Val  Ala  Ser  His  Leu  Gln  Ser
145                      150                      155                      160

Phe  Leu  Glu  Val  Ser  Tyr  Arg  Val  Leu  Arg  His  Leu  Ala  Gln  Pro
               165                      170                      175
```

What is claimed is:

1. A substantially homogenous preparation of N-terminally PEGylated G-CSF or analog thereof, optionally in a pharmaceutically acceptable diluent, carrier or adjuvant, said preparation being essentially free of G-CSF or analog thereof PEGylated at sites other than the N-terminus.

**2.** A preparation of claim **1** wherein the polyethylene glycol used for conjugation has a molecular weight of between about 2 kDa and 100 kDa.

**3.** A preparation of claim **2** wherein said polyethylene glycol has a molecular weight of between about 6 kDa and 25 kDa.

**4.** A preparation of claim **1** wherein said preparation is comprised of at least 90% N-terminally monoPEGylated G-CSF or analog thereof and at most 10% unPEGylated G-CSF or analog thereof.

**5.** A preparation of claim **4** wherein said preparation is comprised of at least 95% N-terminally monoPEGylated G-CSF or analog thereof and at most 5% unPEGylated G-CSF or analog thereof.

**6.** A preparation of claim **1** whererein said G-CSF has the sequence identified in SEQ. ID No. 1.

**7.** A substantially homogenous preparation of N-terminally monoPEGylated G-CSF, optionally in a pharmaceutically acceptable diluent, carrier or adjuvant, wherein: (a) said G-CSF has the amino acid sequence identified in SEQ. ID No. 1; (b) said G-CSF is monoPEGy-lated with a polyethylene glycol moiety having a molecular weight of between about 6 kDa and about 25 kDa.

**8.** A pharmaceutical composition comprising: (a) a substantially homogenous preparation of monoPEGylated G-CSF, said monoPEGylated G-CSF consisting of a polyethylene glycol moiety having a molecular weight of about

6 kDa and about 25 kDa connected to a G-CSF moiety solely at the N-terminus thereof via an amine linkage; (b) fewer than 5% non-PEGylated G-CSF molecules; and (c) a pharmaceutically acceptable diluent, adjuvant or carrier.

**9.** A method of treating a hematopoietic disorder comprising administering a therapeutically effective dose of a preparation of any one of claims **1–8**.

**10.** A method for attaching a polyethylene glycol molecule to a G-CSF molecule, wherein said polyethylene glycol molecule has a single reactive aldehyde group, said method comprising:

(a) reacting said G-CSF with said polyethylene glycol molecule under reducing alkylation conditions, at a pH sufficiently acidic to selectively activate the alpha-amino group at the amino terminus of said G-CSF; and

(b) obtaining the pegylated G-CSF and

(c) optionally, separating the PEGylated G-CSF from from non-PEGylated G-CSF.

**11.** A method of claim **10** wherein said polyethylene glycol molecule has a molecular weight of about 6 kDa to about 25 kDa.

**12.** The PEGylated G-CSF product produced by the process of claim **10**.

* * * * *